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MORPHOLOGY OF REPRODUCTION IN CERATOSTOMELLA FIMBRIATA[1]

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INTRODUCTION

The following report of the results of investigations on a species of the genus Ceratostomella is offered primarily to reveal a process of ascus multiplication that appears to be entirely unique in the field of mycology. This process, while doubtless confined to a relatively small number of the Pyrenomycetes, is regarded as an ordinal character and is presented here as a preliminary to further work in this group. To discuss the subject properly, it is desirable to review the grosser morphological characters of the fungus and to trace the sexual stage from the beginning of perithecial development to the discharge of mature ascospores.

The genus Ceratostomella is grouped with the Sphaeriales of the Pyrenomycetes and includes both parasitic and saprophytic forms. It is well represented among wood-staining fungi of forest trees and lumber yards. *Ceratostomella fimbriata* (Ell. and Hals.) Elliott is important economically as the cause of black rot of sweetpotatoes (*Ipomoea batatas* (L.) Lam.) (*16*).[3] The disease was first described by Halsted (*12*) in 1890, who gave to the fungus the name *Ceratocystis fimbriatum* Ell. and Hals., and the following year Halsted and Fairchild (*13*) published a more thorough description of the life history of the organism.

Owing to the early disintegration of ascus walls, the perithecia were thought to be pycnidia of an imperfect fungus. Saccardo (*23*), still regarding the fruiting body as a pycnidium, gave the first technical description of the organism and transferred it to the form genus Sphaeronema. The first report that 8-spored asci are formed in the perithecia was made by Elliott (*6*) in 1923, and accordingly *Sphaeronema fimbriatum* (Ell. and Hals.) Sacc. became *Ceratostomella fimbriata* (Ell. and Hals.) Elliott. In a later paper attributed to Elliott (*7*) and published after his death, an attempt was made to trace in detail the development of perithecia and asci.

These students, attempting to fit the morphology of Ceratostomella into that of a conventional Ascomycete, did not discover the real character of the sexual stage. An examination of the perithecial contents reveals indeed that there are here features never adequately

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[3] Reference is made by number (italic) to Literature Cited, p. 1077.

accounted for in any treatise on Ascomycete morphology known to the present writers. The need for a reexamination of the development of the black-rot organism has led to the present investigation.

The following observations on the life history of *Ceratostomella fimbriata* were made from material grown on corn-meal and potato-dextrose agars. The endoconidia and early stages in perithecial development were observed satisfactorily in water mounts of live mycelium, but the greater part of the study was made from material fixed in Bouin's picric acid solution, sectioned 4–5μ in thickness, and stained with Flemming's triple combination of safranin, gentian violet, and orange G. The characteristic structures were shown almost equally well in material fixed in Carnoy's and stained with Heidenhain's haematoxylin.

ASEXUAL REPRODUCTION

The process whereby the conidia of *Ceratostomella fimbriata* are abstricted within the sheath of the conidiophore has been discussed at some length by Halsted and Fairchild (*13*), and more recently Lehman (*20*) has contributed some additional facts. The asexual spores of *C. fimbriata* are of two types, oval spores of olive-brown pigmentation and hyaline spores that are mostly linear in shape and extremely variable in size.

Lehman (*20*) presented evidence to show that the walls of the endoconidia were generated anew by the protoplast and were not the result of longitudinal splitting of the conidiophore wall as claimed by Brierley (*2*) for the endoconidia of *Thielavia basicola* (B. and Br.) Zopf. Additional evidence of this fact is adduced here from the frequent presence of an intercalary element between newly formed endoconidia. The structure shown at *a* in Figure 1, G, may consist of lamellar substance that has adhered to the base of the newly abstricted spore. Lehman (*20*) further contended that the thick-walled olive conidia were in no case formed endosporously, since the protoplast is distended from the ruptured tip before the conidium is abstricted. (Fig. 1, C, *a*.) In material with olive conidia formed in great abundance, a considerable number of spores can be found with two distinct walls, the inner wall appearing to be that of an endospore. (Fig. 1, B.) In Figure 1, A, the outer wall of the spore is shown to be continuous with the sheath of the conidiophore, with the line of rupture just visible.

Germination of the thick-walled olive conidia (fig. 1, D), figured by Harter and Weimer (*16*), appears to occur rarely. Hyaline conidia (fig. 1, E to L) are capable of germinating as soon as they are discharged from the sporophore, and water mounts from any area of a colony on agar media will usually show large numbers of germinated and germinating spores which disintegrate after having formed a

EXPLANATORY LEGEND FOR FIGURE 1

A–D.—Olive-brown thick-walled conidia. × 1,500. A and B indicate that the first abstricted spore is double walled; spores produced later from the same conidiophore are single walled.
E–L.—Hyaline conidia.
E.—Conidiophore showing hyphal fusion at its base. × 1,000.
F.—Separation of the spore wall from the conidiophore end wall at *a* as a result of plasmolysis. × 1,500.
G.—Probably a remnant of the middle lamella (*a*) shown at base of recently abstricted spore. × 1,000.
H, I.—Germinated spores forming new endoconidia. × 1,000.
J.—Early stage in germination. × 1,000.
K, L.—Evacuation of spore contents after germination; contrast with H. × 1,000.
A–D and F from stained sections, others from live material.

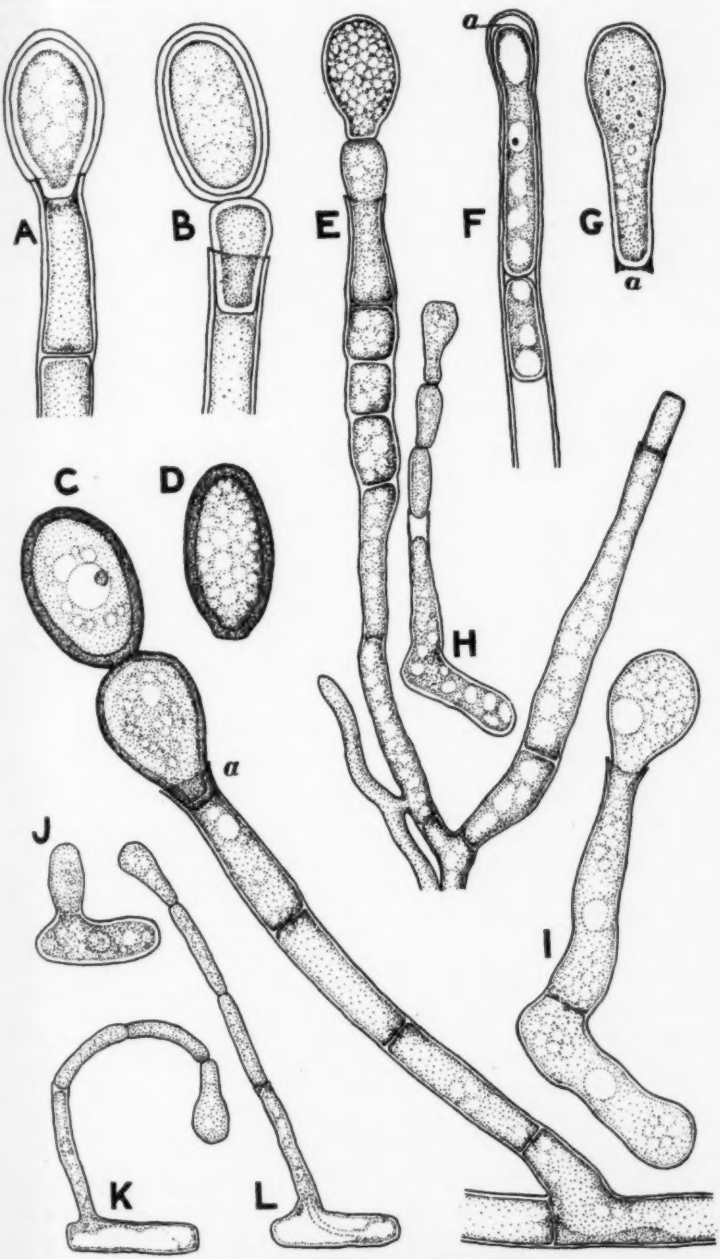

FIGURE 1.—Endoconidia of *Ceratostomella fimbriata*
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chain of smaller endoconidia. There is normally no evacuation of spore contents as appears in Figure 1, K, L. The one or more germ tubes form at a characteristic angle to the conidium (fig. 1, H to L), and may proceed at once to the formation of new endoconidia (fig. 1, H).

In Petri-dish cultures and on sweetpotato roots in moist chambers, the aerial chains of conidia collapse and become suspended in droplets of water which condense among the aerial hyphae. Under these conditions large numbers of the hyaline spores germinate and the germ tubes grow downward to the surface of the medium. Conidia discharged beneath the surface of the agar germinate and grow upward, discharging new endoconidia above the surface of the medium. Germinating conidiospores very frequently fuse with one another and with neighboring hyphae, forming an irregular entanglement of aerial mycelium having considerable rigidity.

INITIATION AND DEVELOPMENT OF PERITHECIA

Mature perithecia of *Ceratostomella fimbriata* are black, membranaceous, and appear dark brown under magnification. The long beak or perithecial neck may reach a length of 1 mm. (Fig. 2, A.) Perithecia frequently occur on the host under field and storage conditions but are formed more abundantly on agar plate cultures. They are normally superficial, sometimes partially immersed, and frequently inclosed in an almost stromatic growth of conidiophores and germinating conidia. (Fig. 2, B.) Perithecia are formed more abundantly in certain cultures than in others, but a large number of single ascospore isolations have failed to reveal any evidence of sexual strains. The antheridium and oogonium are borne on the same hyphal branch. Two or more perithecium initials are occasionally involved in what later develops into a single perithecium (fig. 3, P), but such a coalescence of two or more perithecia may occur at any later stage in development and has no sexual significance. (Pl. 1, A.) Many types of hyphal fusions and fusions of conidia with mycelium occur in all cultures and are regarded as having no relation to sex. The fungus is, therefore, considered to be completely self-fertile, or perhaps parthenogenetic, since the antheridium does not appear to function in fertilization.

The perithecium initial is earliest recognized as a hyphal branch with a thin-walled and slightly recurved hyaline tip and with one or more short hyaline lateral branches. (Fig. 3, A to O.) The perithecial stalks appear to be identical with those structures which ordinarily develop into conidiophores. All cells involved in the development of the perithecium initial are uninucleate. The recurved terminal portion divides into three cells (fig. 3, H, J, Q), the middle or subterminal one of which becomes the oogonium. This very characteristic 3-cell arrangement is distinguishable in water mounts of fresh material as well as in stained sections.

EXPLANATORY LEGEND FOR FIGURE 2

A.—Mature perithecium. × 250.
B.—Photomicrograph of section through an aggregation of perithecia in various stages of development. × 100.
C.—Photomicrograph of section through a young perithecium, showing oogonium in its initial binucleate stage. × 530.
D.—Same, with oogonium in 4-nucleate stage. × 530.
E.—Same, after the oogonium has divided into 2 binucleate cells. × 530.

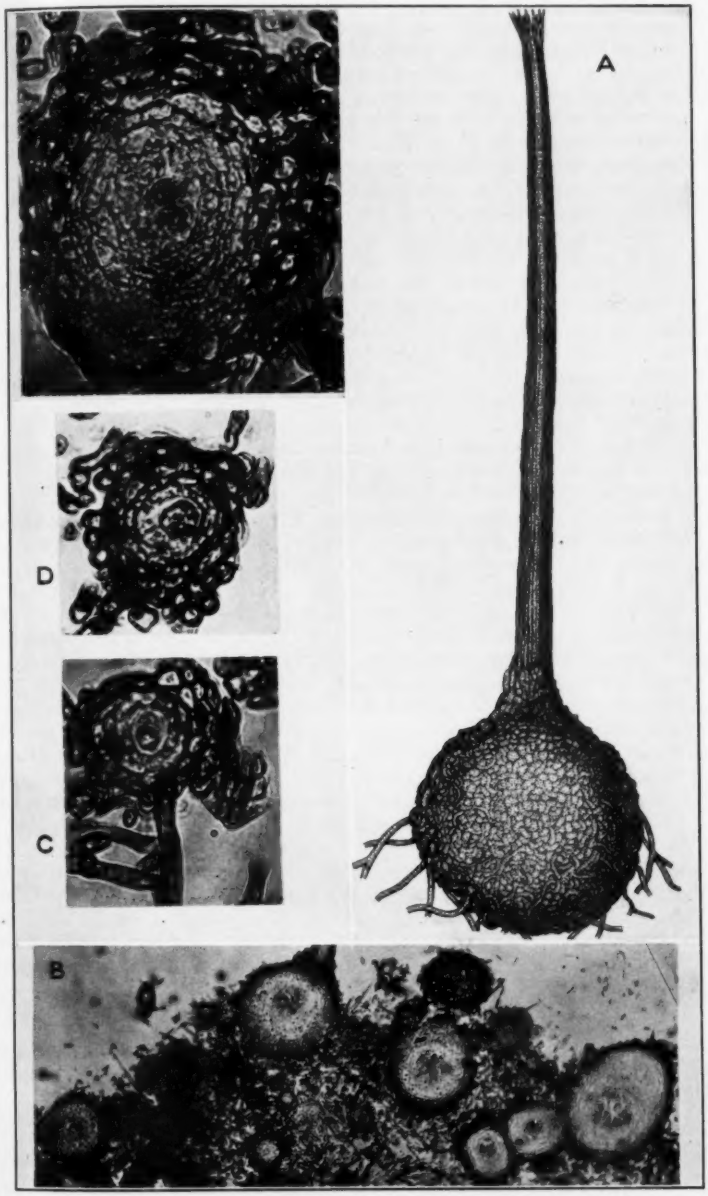

FIGURE 2.—Perithecia of *Ceratostomella fimbriata*

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The antheridium is the first lateral branch formed below the oogonium. (Fig. 3, A to C, I to O.) Structurally it is identical with the branches formed later from the same and lower nodes of the perithecial stalk. (Fig. 3, R, S.) It is strange that the recurved oogonium should display such intimate contact with this particular organ, for apparently a fusion between the two structures rarely if ever occurs. Certainly there is no proof from the writers' preparations that a nucleus from the antheridium passes into the oogonial cell, either directly or through the terminal cell. On the other hand, there is evidence that a nucleus from the terminal cell of the ascogone may migrate into the oogonium, a phenomenon comparable to that believed by Faull (*8*) to occur in *Laboulbenia chaetophora* Thax.

It has been observed in two instances that two nuclei are present in the terminal cell at a very early stage. (Fig. 3, Q, and fig. 4, A.) This might suggest that the terminal cell acts as a trichogyne in conjugation with the antheridium, but evidence of such a conjugation is entirely lacking. The few clear cases showing an actual fusion appear to involve sterile structures and are unaccompanied by nuclear activity. (Fig. 3, V to X.) Figure 4, A, especially might be adduced as evidence that the oogonium is fertilized by a nucleus from the terminal cell. Here the wall separating the tip cell from the oogonium is incomplete, while the two identical nuclei in the tip cell are probably the result of a recent nuclear division. In later stages a single nucleus is found in the tip cell, which frequently persists without loss of turgor for some time after the paired nuclei are present in the oogonium. (Fig. 3, T, and fig. 4, B, F.)

The presence of a 3-celled structure previous to the origin of the paired nuclei, as shown in Figure 3, H, J, should be emphasized for the reason that it resembles very closely the supposed 3-celled ascogone of *Sphaerotheca castagnei* Lév., which, according to Hein (*17, p. 400 and fig. 38*), is formed after fertilization. These structures in *Ceratostomella fimbriata* can be seen in live material before there has been any possible fusion of oogonium with antheridium; and if the 3-celled ascogone is truly a sporophytic growth, it must be concluded that the origin of the paired nuclei does not depend upon the migration of a nucleus from an antheridium.

In Figure 3, Y, is represented a curious and conspicuous structure that is characteristically present at an early stage in perithecial development. Its appearance at once suggests a trichogyne. Perhaps it is a vestige of an ancestral copulatory organ, comparable to the trichogyne of some alga or lichen. On the other hand, were it not for the fact that the organ (fig. 3, Y, *a*) is so frequently present at just

EXPLANATORY LEGEND FOR FIGURE 3

A–H.—Initial organs of perithecial stalk drawn from live material; *a*, antheridial, and *b*, oogonial branch. × 700.

I–O.—Initial organs drawn from stained sections. The terminal recurved portion in each case is the oogonial branch. × 1,000.

P.—Two perithecial initials involved in a single coil. × 1,000.

Q.—Perithecial initial showing 3-celled ascogone. Fertilization results from the migration of one nucleus from cell *b* into the oogonium, *a*; antheridium probably at *c*. × 1,000.

R, S.—Later stage, showing first outgrowth of hyphae that envelop the oogonium. × 1,000.

T.—Perithecial initial showing 3-celled ascogone with first layer of enveloping hyphae; oogonium shown at *a*, with tip cell (*b*) and basal cell of ascogone (*c*). × 1,000.

U.—Young perithecium showing oogonium (*a*) after first nuclear division. × 1,000.

V, W.—Aborted perithecia; cell fusions at *a* involve sterile structures. × 1,000.

X.—Same; *a* and *b* show adjacent sections of same structure. × 1,000.

Y.—Perithecial initials at *a*, *b*, and *c*, showing an accessory organ curiously suggesting a trichogyne. × 1,000.

this stage, it might be regarded as simply a modified or aborted conidiophore, such as may frequently be seen growing from the superficial layers of older perithecia. It seems sufficiently clear that this appendage is not concerned with fertilization. The structure can not be identified in later stages and is apparently absorbed in the growing perithecium.

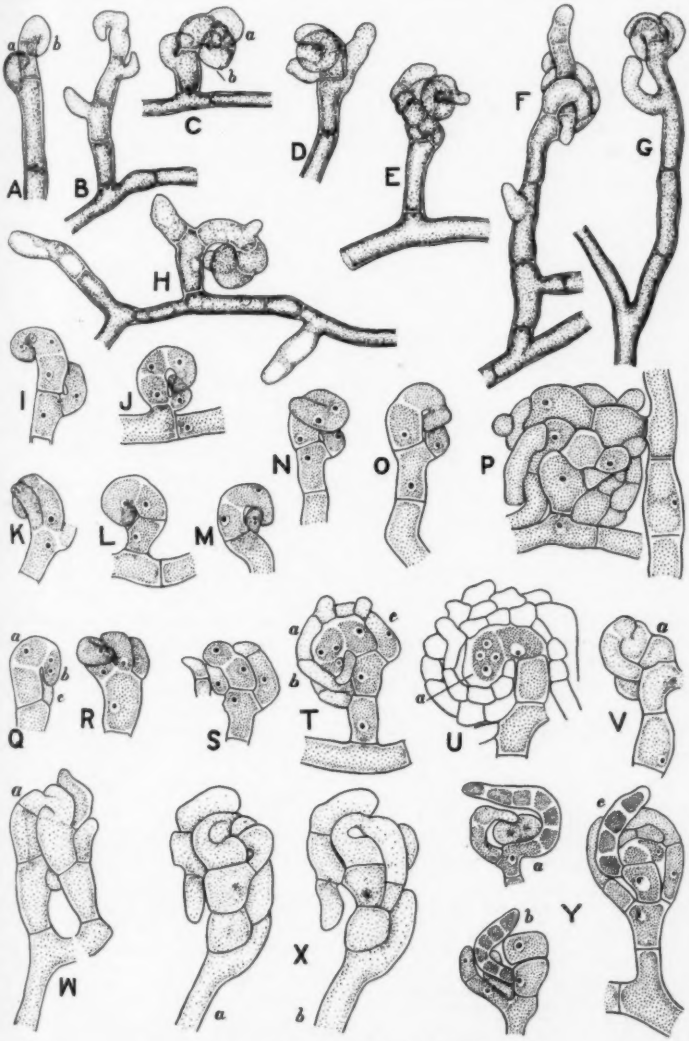

FIGURE 3.—Initiation of perithecia in *Ceratostomella fimbriata*
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MULTIPLICATION OF ASCI WITHIN THE PERITHECIUM

As the perithecium enlarges externally by new hyphal growth the inner cavity enlarges, apparently by digestive action. (Fig. 4, I, and fig. 5.) Dissection of a live perithecium reveals activity in the cavity resembling yeast or bacterial fermentation in such a striking manner that the presence of a parasite was at first suspected. Later observations led to the discovery of a method of ascus multiplication that, judging from previous accounts of Pyrenomycete development, appears to be peculiar to *Ceratostomella fimbriata*.[4] In the following paragraphs an attempt will be made to trace the course of development of the oogonium and of the cells that result from its division.

The subterminal cell of the perithecial stalk becomes binucleate before any enveloping hyphae are in place, and this binucleate condition persists up to the time of ascus formation. No nuclear fusion occurs in the oogonium, as claimed by Harper and others (*11*, *15*, *17*) for other Ascomycetes. The only fusion of nuclei occurs in the young asci.

The oogonium forms a core for the young fruiting body (fig. 3, T), the nuclei and cell plasm become separated from the wall, and the protoplast lies free in the cavity of the perithecium (fig. 4, B, C). The protoplast remains undivided for a brief time after the first layers of enveloping hyphae are in place. (Fig. 2, C, D; fig. 3, T; and fig. 4, B to D.) Nuclear divisions then occur, resulting in a cell with four to eight nuclei. (Fig. 3, U, and fig. 4, E to H, J, K.) The 4 to 8 nucleate body then undergoes a division or fragmentation (fig. 2, E; fig. 4, I, L; and fig. 6, A to C), and further division of the daughter cells and their progeny results in a multitude of apparently naked cells embedded in a nutrient medium and entirely detached within the enlarging perithecial cavity (fig. 5, and fig 6, D).

The division of the daughter cells, following fragmentation of the oogonium, takes place in the binucleate condition (fig. 6, E, *a*), so that cells with four nuclei are frequently found before the constriction is complete (fig. 6, E, *b* to *d*). Later, certain of these cells fail to undergo any further division; the two nuclei fuse and the cell enlarges to form a young ascus. (Fig. 6, F, G, H.) The various stages in the nuclear fusion, as shown in Figure 6, J, are observed

[4] Some cytological preparations have been made of *Ceratostomella* (*Endoconidiophora*) *coerulescens* (Münch) Clements and Shear, and these indicate that an essentially similar method of ascus multiplication occurs in this species.

EXPLANATORY LEGEND FOR FIGURE 4

A.—Perithecial initial showing fertilization occurring simultaneously with the appearance of the first enveloping hyphae; constriction cutting off the tip cell from the oogonium at *a* (incomplete).
B.—Young perithecium showing 3-celled ascogone after the tip cell (*b*) is separated from the oogonium (*a*).
C.—Same stage as B; either the tip cell is not shown or in this case it has become the oogonium.
D.—Early stage with the old hyphal wall still intact about the oogonium at *a*; terminal cell of ascogone not shown.
E.—Later stage, after nuclear divisions have occurred in the oogonium.
F.—Early stage with the three cells of the ascogone still visible. The significance of the apparently degenerate nuclei in the oogonium at *a* is not clear.
G.—Young perithecium after first nuclear division of the oogonium.
H.—Same, showing oogonium just before cell division.
I.—Same, following the first fragmentation.
J.—Oogonium in 4-nucleate stage.
K.—Oogonium with eight nuclei previous to fragmentation.
L.—Ascogenous cells resulting from division of the oogonium. A nuclear division has occurred in the daughter cell *a*, and cell *b* shows nuclei in process of division. Dotted lines in J, K, and L indicate the limits of the perithecial cavity.
All figures × 1,000.

regularly and clearly. The remaining cell of the recently divided pair will undergo a further division (fig. 6, F, *c*), and one of its progeny will form an ascus. It is natural to assume that each daughter cell normally receives nuclei of diverse lineage. The only evidence of the relationship of paired nuclei must be based upon their observed conjugate division and their relative positions at the time of fixation, and upon this basis it can not be said with certainty that the two nuclei which fuse in the ascus are not sister nuclei.

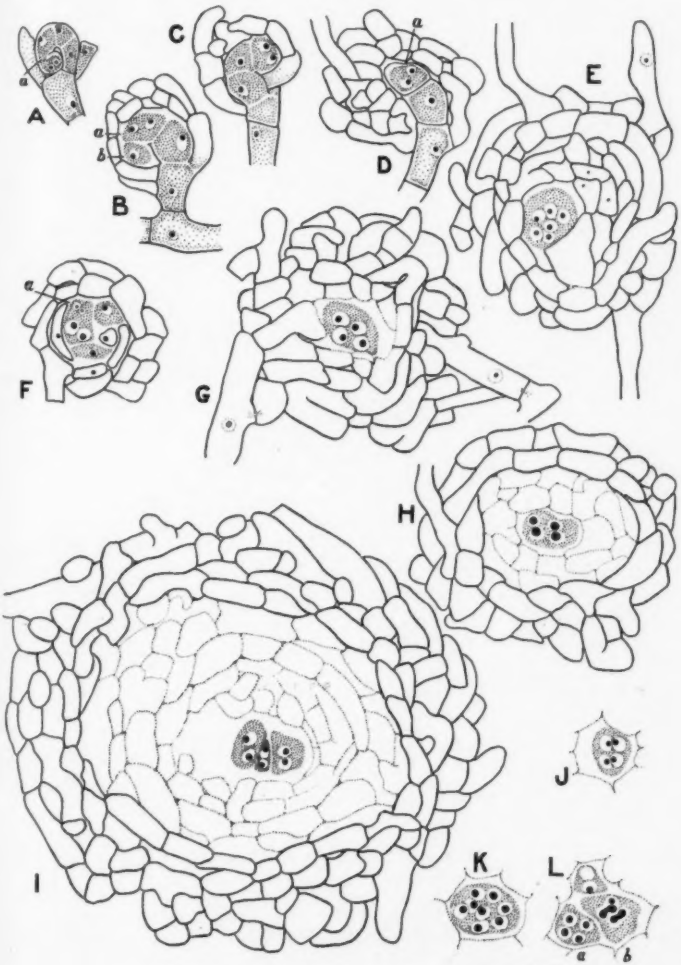

FIGURE 4.—Development of perithecia in *Ceratostomella fimbriata*

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Free cells of various sizes with 1, 2, and 4 nuclei can be found intermingled in the perithecial cavity even before the fruiting body is half mature. (Fig. 5.) These young ascogenous cells may cling together in series of two to four, but they are without hyphal connection with the wall of the perithecium. In those cases in which

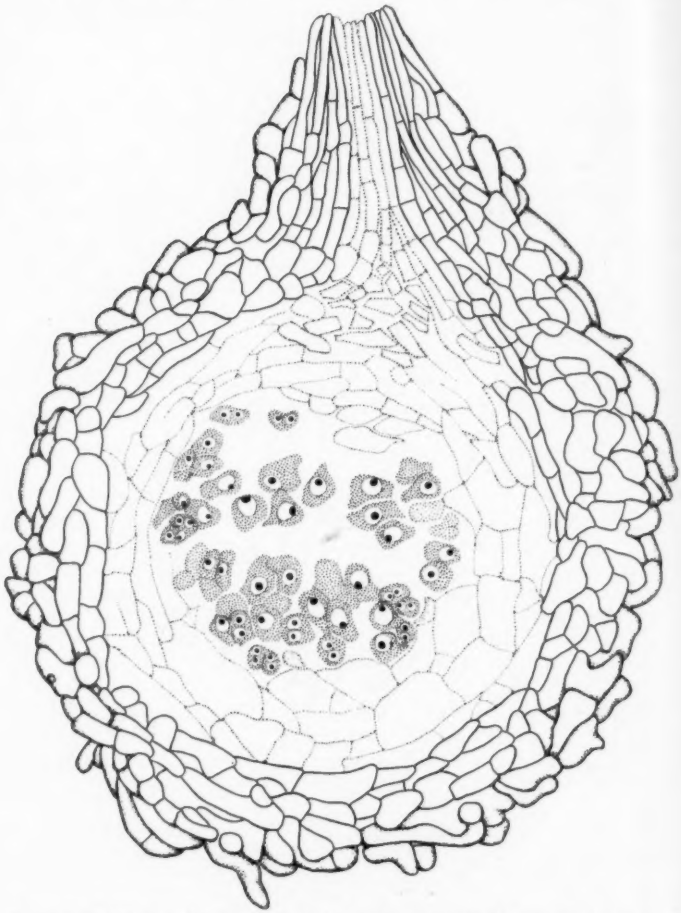
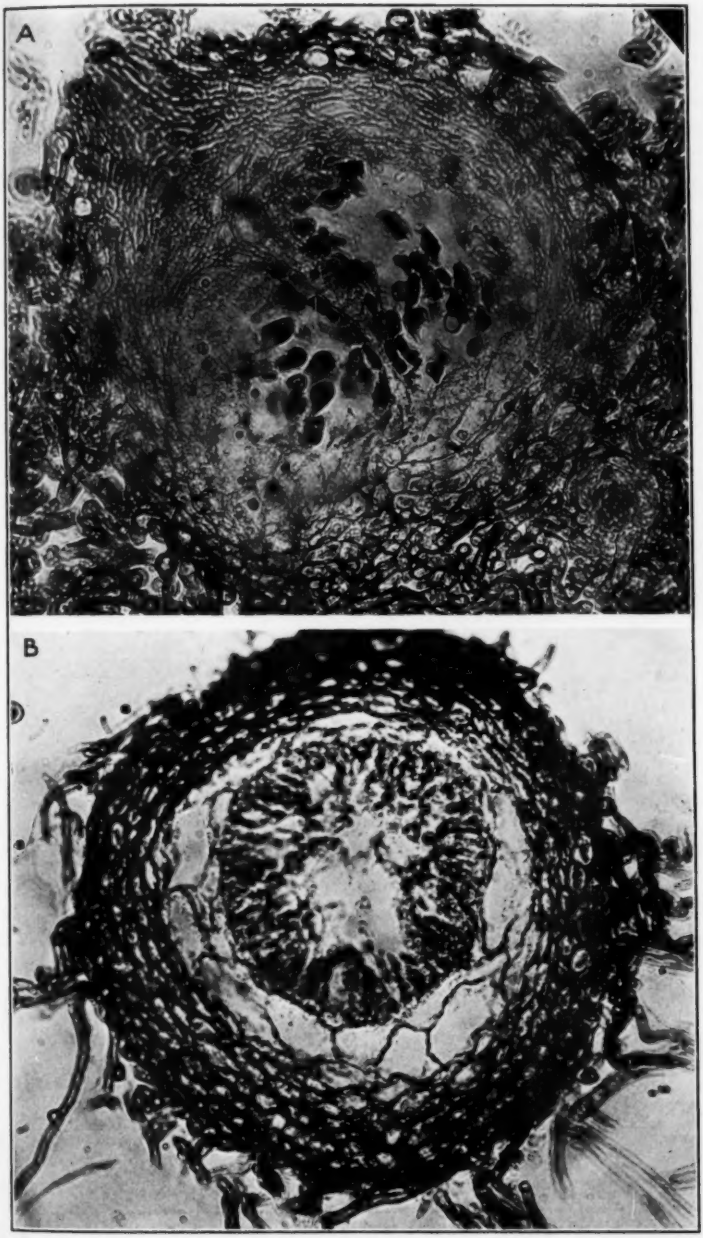


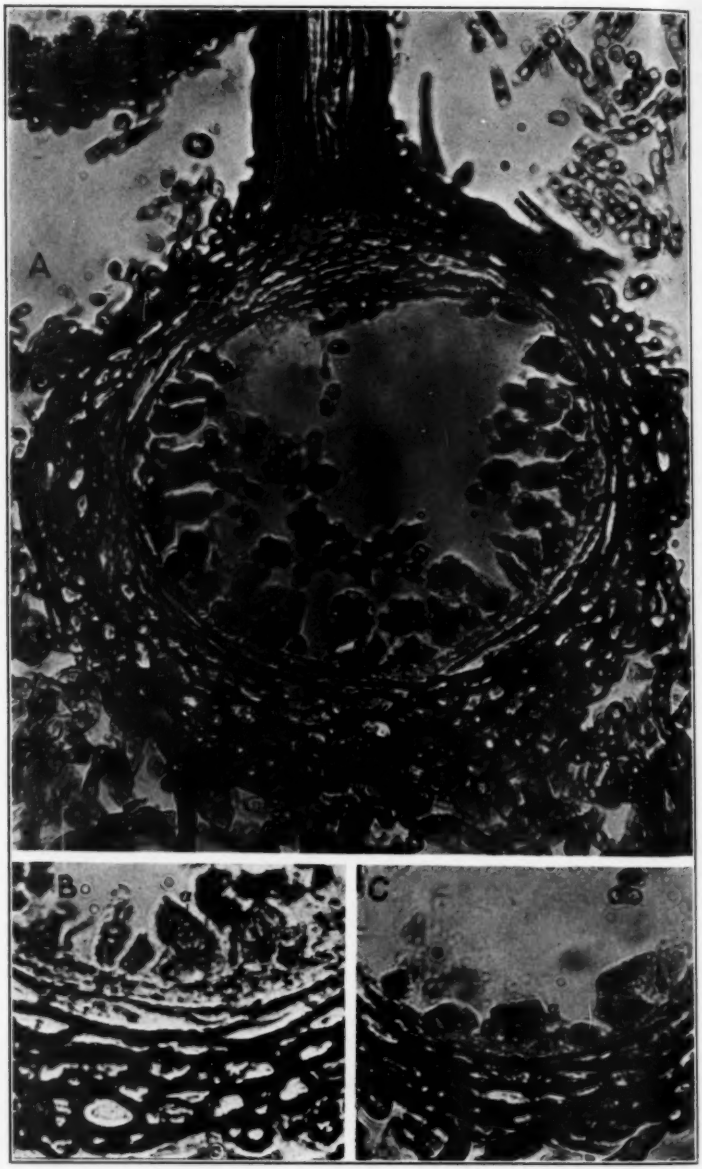
FIGURE 5.—Perithecium of *Ceratostomella fimbriata* at beginning of beak formation, showing zonation of perithecial walls and arrangement of ascogenous cells in the cavity. × 700

cells cling together they do so by a very slender filament. (Fig. 6, F, *d*.) The connected cells frequently form short chains not wholly unlike the series of asci described by Faull (*8, fig. 38, pl. 38*) for some of the Laboulbeniales.

Contrary to claims made by Elliott (*7*), no structures resembling ascogenous hyphae are found at any stage in the development of



Perithecia of Ceratostomella: A, Photomicrograph of section showing coalescence of immature perithecia of *C. fimbriata;* the original wall of separation is nearly consumed by digestive processes in the two cavities. × 400. B, Photomicrograph through section of immature perithecium of *C. adipcsum* showing radially disposed columns of ascogenous cells. × 400.



Arrangement of asci in perithecia of *Ceratostomella fimbriata:* A, Mature fruiting body with most of contents collapsed against the walls of the perithecium. × 500. B, C, Detail showing asci with inner vesicle at *a*. × 660.

the perithecium. Instead of forming ascogenous hyphae, the protoplast of the oogonium breaks up into binucleate ascogenous cells which after a series of further divisions develop into asci. (Fig. 6.) The absence of cell walls and of cross walls permits the dividing protoplasts to separate easily. (Fig. 6, G.) Thus for the most part the numerous cells are wholly detached from one another but frequently remain in apposition, forming irregular series of two to four cells. (Fig. 6, F, G, H.)

The first stage of division of the ascogenous cells is characterized by the appearance of a simple cleft, as shown in Figure 6, E. The fissure increases until the protoplasmic connection is broken. (Fig. 6, G.) The cells in process of division form structures resembling ascus hooks, or what are often referred to as "croziers" (*26*). (Fig. 6, E.) It is clear that Varitchak (*26*) may easily have been misled into supposing that true ascus hooks are formed in *Ceratostomella piceae* Münch. With few exceptions his figures show structures that appear identical with those found in *C. fimbriata*, in which there can be no doubt as to the mechanism of the dividing cells. There are no ascogenous hyphae with croziers, but simply free cells dividing by a peculiar process of constriction. (Fig. 6, E.) The type of septation shown in Varitchak's Figure 2, *g*, Plate 10, has not been observed in *C. fimbriata*, although it is not inconceivable that a division might occasionally take place in such a fashion. His figures do not indicate that this is the usual procedure in *C. piceae*. In *C. fimbriata*, a daughter cell is rarely abstricted with a single nucleus, leaving three nuclei in the parent cell. (Fig. 6, I, *b*.) A cell is sometimes found dividing as shown in Figure 6, I, *c*, where an extra nuclear division has taken place in advance of the separation of the daughter cells. Such irregularities in cell mechanics might well be anticipated.

After the initial stage the perithecial wall assumes two well-marked zones—an inner zone of very thin-walled colorless cells that are being rapidly digested, and an outer zone of relatively thick-walled pigmented cells where new layers are being constantly added. (Fig. 5.) Neither paraphyses nor periphyses are found at any stage in the development of the perithecium.

The smallest and most recently formed ascogenous cells are found normally in close proximity to the lining of the cavity. (Pl. 2, A.) However, they are disposed at all angles and are often found in the center, remote from the perithecial wall. (Fig. 5.) It is important to note that the digestion of the inner wall of the perithecium appears to take place equally in all directions, so that there is no possibility of the central core of ascogenous cells retaining their original connection with the crown of the perithecial stalk. There is thus entire absence of hyphal connection with the body of the perithecium, except, perhaps, as may rarely result through secondary attachments of the isolated protoplasts. The semiliquid débris resulting from the recently digested inner cells of the perithecium forms an excellent medium for orientation of the unwalled cells. The absence of a cell wall possibly facilitates the nutritive relation of the ascogenous cells with the medium in which they are embedded. This apparently naked condition begins with the single-celled oogonium and is maintained until the asci are in process of maturation, at which time a new wall is formed endogenously, as will be shown later.

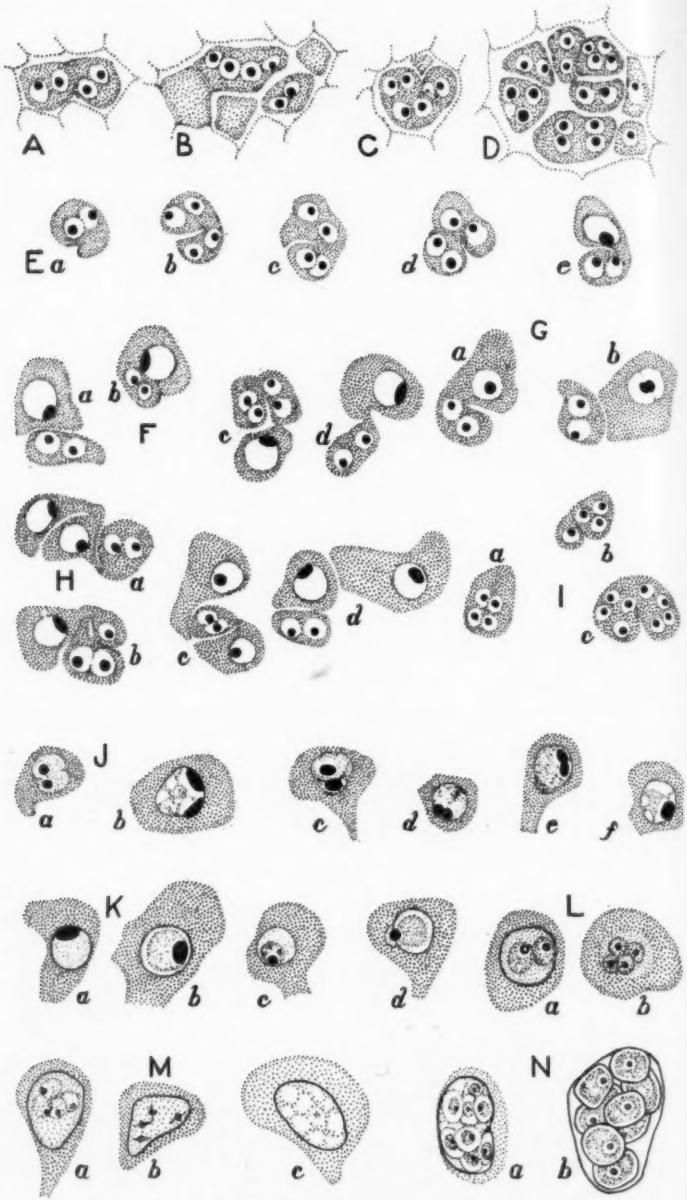


FIGURE 6.—Division of ascogenous cells and maturation of asci in *Ceratostomella fimbriata*

For explanatory legend see opposite page

The structure of the young asci and of the dividing cells, as shown in Figure 6, A to M, can not readily be seen by dissecting the live perithecia in water. The tiny, unwalled, and almost colorless cells become dissolved or lose their form completely in water. But they can be demonstrated clearly by crushing or dissecting the perithecia in Amann's solution with nigrosine as a stain.[5] This solution both kills and fixes the material, and the naked protoplasts stain a faint blue with the nigrosine.

The chains of ascogenous cells in *Ceratostomella adiposum* (Butler) Sartoris appear as simple columns of two or more cells regularly disposed about the interior of the perithecium as described by Sartoris (*24*). An examination of some of Sartoris's slides and of fresh material of *C. adiposum* confirms his observation that asci are formed by a simple terminal abstriction of binucleate cells. Sartoris places no emphasis on the fact that these columns of ascogenous cells are not growing out of the wall of the perithecium or from any basal hyphae lining the cavity, but are, like the ascogenous cells of *C. fimbriata*, disposed unattached within the perithecial cavity and are in fact growing *toward the periphery* of the perithecium. (Pl. 1, B.) The ascogenous cells appear to consume the body of the perithecium by continual growth from the center outward. (Fig. 5; pl. 1, A; and pl. 2, A.) In other words, the inner wall of the perithecium is rapidly digested, perhaps by an enzymatic action on the part of the rapidly multiplying free cells, which continuously encroach upon the limits of the cavity.

MATURATION OF ASCI

The first asci begin to mature at about the time the perithecium has attained approximately one-half its full diameter and when the beak or neck of the perithecium has just begun to form. (Fig. 5.) From this time on, ascogenous cells in all stages of development can be seen in the same perithecium, the younger cells prostrate and lying at all angles near the wall of the cavity.

The preponderance of asci in the fusion nucleus stage (fig. 6, J, *f*) indicates that this stage persists for a relatively long period, while the subsequent 2, 4, and 8 nucleate stages occur in rapid succession. The nuclear behavior from the beginning of the first division to the complete delimitation of ascospores is very obscure; only rarely can

[5] The following formula was used in this work: 1 part concentrated lactic acid, 1 part phenol, 1 part 95 per cent alcohol, 2 parts glycerin. Add a few crystals of nigrosine.

EXPLANATORY LEGEND FOR FIGURE 6

A–D.—Early stages in the fragmentation of the oogonium. Cells with four nuclei are prepared for another cell division. Dotted lines indicate limits of perithecial cavity. (Refer to fig. 4, I.)

E.—Stages in division of ascogenous cells; *a*, before nuclear division; *b–e*, after nuclear division, showing separation of daughter cells; two nuclei in upper cell of *e* have fused in forming a young ascus.

F, G.—Further proliferation of ascogenous cells; in each case one daughter cell forms an ascus while the second cell prepares for another division.

H.—Linkage of asci and ascogenous cells after successive divisions.

I.—Irregularities in cell division; *a* is probably the result of a division such as is shown in *c*; *b*, unequal division.

J.—Nuclear fusion in the ascus (*a–e*); young ascus (*f*).

K.—Nuclear contraction in the ascus; *a*, before contraction; *b–d*, stages in contraction showing origin of the ascus vesicle. It appears in *d* that the nucleolus is being ejected from the vesicle.

L.—First nuclear division in the ascus; wall of the vesicle distinct in *a* and very faint in *b*.

M.—Further expansion of the nuclear membrane to form the wall of the ascus; 4-nucleate stage in *a*, faint spindle-shaped structures in *b*; *c* indicates the obscure nuclear behavior during spore delimitation.

N.—Maturation of ascospores; *a* shows ascus and eight young spores with outer integument of cytoplasm still adherent to the ascus; *b*, ascus with eight mature spores.

All figures × 1,500.

the several stages shown in Figure 6, L, M, be seen clearly, the outline of the vesicle alone remaining distinct. Most frequently the asci have the appearance indicated in Figure 6, M, *c*, from which it would appear that the nuclei during this period do not usually enter a resting stage until the eight spores are formed.

The first nuclear division in the ascus is characterized by the development of an ascus vesicle, the wall of which appears to be the membrane of the fusion nucleus. (Fig. 6, L, *a*.) All three divisions and the later delimitation of ascospores, therefore, take place within the nuclear wall. (Fig. 6, K to N, and pl. 2, B, C.) In some cases the first indication of the ascus vesicle is the contraction of nuclear substance, resembling a nuclear plasmolysis. (Fig. 6, K, *b*, *c*.) In Figure 6, K, *d*, there is evidence that the nucleolus is ejected from the vesicle just preceding the first nuclear division, but there is no reason to conclude that this is the usual procedure. The regular disappearance of the nucleolus may be accounted for in some other fashion. That the nucleolus at this stage corresponds to a plasmosome (*27*) is indicated by its affinity for plasma stains rather than chromatin stains, and the possibility should not be overlooked that it may perform some active function in the formation of the vesicle wall and of the cytoplasm within the vesicle.

The vesicle expands rapidly and continuously during the period of nuclear divisions (fig. 6, L, M) and has reached nearly the dimensions of an ascus by the time the eight young spores are formed (fig. 6, N, *a*). The ascospores undergo a further increase in size, and this expansion usually succeeds in rupturing the membrane, leaving the spores free within the perithecium, from which they are ejected through the beak in a mucilaginous coil. The extreme fragility of the mature ascus wall is indicated by the fact that asci can rarely be found with a full complement of eight mature spores. (Fig. 6, N, *b*.) Those asci observed by dissection of perithecia in water have partially broken walls or contain immature spores.

So far as the writers are aware, no one has reported previously a case in which the wall of the fusion nucleus becomes the wall of the ascus. The procedure is doubtless peculiar to those species of Pyrenomycetes whose asci in their younger stages are without a definite wall. The nuclear contraction just preceding the first division is perhaps comparable to the contraction phase frequently described in the literature on ascus cytology, but in such cases the nuclear membrane does not persist as in *Ceratostomella fimbriata*. In regard to the nuclear divisions in the ascus, Elliott (*7, p. 421*) observed that "these divisions occur within the nuclear-plasm, which in many cases is distinctly separate from the cytoplasm of the ascus." He does not account for the origin of the ascus wall, doubtless for the reason that he failed to observe the essentially naked condition of the young asci.

In describing the life history of the red alga *Polysiphonia violacea* Grev., Yamanouchi (*28, v. 41, p. 431*) writes as follows:

> It is a remarkable fact that the membrane of the original nucleus in the tetraspore mother-cell persists through the two mitoses which have just been described. The area included by this membrane increases in size with the growth of the cell.

In a later paper Yamanouchi (*28, v. 42, pl. 27*) illustrates this stage in the development of the tetraspore mother cell of Polysiphonia.

The phenomenon apparently has little relation to the origin of an ascus wall in Ceratostomella.

Sartoris (*24*) apparently overlooked the occurrence of ascus vesicles in *Ceratostomella adiposum*, although they appear clearly on his slides. In a personal discussion of this point Sartoris expressed himself as favorably disposed to the interpretation that an ascus wall is formed endogenously in *C. adiposum*. It is true that a vesicle appears to be absent in many of the developing asci in both *C. fimbriata* and *C. adiposum*, but it also appears that many groups of ascospores mature in complete absence of a retaining wall. It was this condition which probably misled mycologists for a long time in supposing that the perithecia of these fungi were pycnidia, producing numerous free pycnidiospores.

DISCUSSION

An attempt has been made to throw additional light on a much neglected field of fungus morphology—early perithecial development in the Sphaeriales. The results of a previous study on the development of *Ceratostomella fimbriata* by Elliott (*7*) indicate that the organism conforms to the familiar views already held regarding Ascomycete morphology. The present investigations, however, do not support Elliott's conclusions in many particulars.

In the material studied by the writers it is clear that both antheridium and oogonium are borne on the same hypha. (Fig. 3, A to O.) If a conjugation takes place at all it is between structures genetically separated by not more than three cell divisions. It is believed, however, that fertilization in *Ceratostomella fimbriata* is accomplished by the migration of a nucleus from the terminal cell of the ascogone into the subterminal cell or oogonium. (Fig. 3, I, Q, and fig. 4, A.) In those species of Ascomycetes where a union of male and female elements can be demonstrated by the methods of hybridization, the question of fertilization to that extent may be regarded as closed (*4*, *22*); but in those species, such as *C. fimbriata*, where a like method appears unavailable, no positive conclusions can be reached. Even though a conjugation of two elements can be adequately demonstrated, proof is yet lacking that this constitutes an effective sexual act.

Dangeard (*3*) speaks of the antheridium of some Pyrenomycetes as a "trophogone," inferring thereby that a fusion of a "trophogone" with an oogonium has only a nutritive value. However, convenience may justify the use of the term "antheridium," although it is supposed, in the absence of proof of nuclear migration from antheridium to oogonium, that a fusion of these structures is not sexually effective. In the case of *Ceratostomella fimbriata* there is no proof that antheridium and oogonium ever fuse, although the two structures can be easily identified at the stage where such a fusion would be expected to occur. Yet there seems to be no reason to doubt the numerous reports of Harper and his students wherein a true fertilization is described for a number of species of Ascomycetes. Attention should be called only to the situation in many Phycomycetes where development of the oogonium proceeds whether or not copulation takes place (*5*, *9*).

The absence of a nuclear fusion in the oogonium of *Ceratostomella fimbriata* may indicate further that the paired nuclei have not resulted

from copulation but have developed spontaneously in the terminal region of the ascogone. (Fig. 4, A.) Gwynne-Vaughan and Williamson (*11*) have recently reaffirmed a conviction that nuclear fusions occur following conjugation in Pyronema. It is a familiar fact, however, that nuclear fusions at this stage have been seriously questioned by various observers, even in Pyronema.

The oogonium of *Ceratostomella fimbriata* may be compared to that of species of Endomyces, or to that of *Sphaerotheca castagnei*. In *Endomyces magnusii* Ludwig (*10*) the oogonium develops directly into a single ascus. In *S. castagnei* (*14*, *17*) the oogonium by division forms a series of three to five cells, only one of which normally becomes an ascus. In *C. fimbriata* the oogonium, by its division and by further division of its progeny, forms a multitude of cells all destined to become asci.

Three unique features in the process of ascus multiplication in *Ceratostomella fimbriata* deserve particular emphasis. One consists in the complete absence of any hyphal connection between the body of the perithecium and the ascogenous cells that occupy its interior. (Fig. 5.) Such a wholly unorthodox situation appears less remarkable when it is recalled that the ascogenous cells are not derived from ascogenous hyphae but result from a progressive cell fission that begins with the original protoplast in the subterminal cell of the ascogone. (Fig. 6, A to D.) We need further to recall that the need for connecting hyphae in obtaining nutrients is obviated by the condition that the ascogenous cells are themselves embedded in a nutrient medium.

Varitchak (*26*) speaks at considerable length of the ascogenous hyphae in *Ceratostomella piceae*, whereas his figures show practically no evidence of these structures, and in view of the observations made with respect to *C. fimbriata* it appears doubtful if ascogenous hyphae are formed in *C. piceae*. Varitchak regards the crescent-shaped and recurved cells in the perithecium of *C. piceae* as ascus croziers, supposing that the penultimate portion becomes an ascus while the ultimate and antepenultimate cells fuse to form a second one, such being the manner in which a crozier is supposed to function. Nevertheless, his figures indicate that asci may be formed by a simple cell fission essentially identical with the process in *C. fimbriata*.

The division of ascogenous cells in *Ceratostomella adiposum* differs from that in *C. fimbriata* only in appearance. In both species there is a simple fission of binucleate cells. In *C. fimbriata* the progress of fission produces curious structures superficially resembling ascus croziers, and the manner in which the young asci cling together (fig. 6, H) suggests the serial arrangement of asci in *Laboulbenia chaetophora* (*8*). In *C. adiposum* such hooked structures are seldom found; the dividing cells remain in apposition along the entire plane of fission, so that neat rows of cells are formed that stand side by side and give a false appearance of a hymenium lining the whole inner face of the perithecium. (Pl. 1, B.)

A second feature of particular interest is the apparently naked condition of the ascogenous cells and young asci. The absence of a cell wall begins with the primary ascus or oogonium (fig. 4, G) and appears first as a plasmolysis or shrinkage of the protoplast away from its wall. The delicate membrane that was once the hyphal wall can

still be seen as indicated in Figure 4, D, *a*, but from this point on the tiny protoplasts multiply in an essentially naked condition until a new wall is formed endogenously in the ascus. (Fig. 6, K to N.) There is no indication that the protoplasts may be bounded by a plasma membrane of a more definite nature than a simple surface film. The frequently angular shape of the dividing cells indicates a considerable degree of firmness or rigidity of the protoplasm independent of any surface film. The intimate contact of the unwalled cells with the semiliquid débris resulting from the digestion of the inner cells of the perithecium must eliminate any need for hyphal connections with the base of the fruiting body.

Another feature in the life history of *Ceratostomella fimbriata* which is unknown among other ascomycetous fungi so far studied is the endogenous formation of an ascus membrane. By this process the wall of the fusion nucleus becomes the wall of the ascus. Among the Ascomycetes that have been studied cytologically, it has been frequently reported that the spindles are formed within the nuclear wall during the meiotic divisions. In no case, however, has it been shown that the original membrane of the fusion nucleus persists through the three divisions and finally incloses the eight ascospores, although this appears to be the usual procedure in *C. fimbriata*. (Fig. 6, K to N.) If one considers that all the young asci here have developed in the absence of a wall, it may appear that such a persistence of the nuclear membrane has a peculiar utility for this and related species.

There are certain theoretical objections to the view that the ascospores are delimited within the membrane of the fusion nucleus. The interpretation does not accord easily with the view that cytoplasm and nucleoplasm are multiplied independently of each other. It does accord with the view that cytoplasm may be a product of nuclear activity. It is of course possible that the wall of the vesicle (fig. 6, L, and pl. 2, B, C) did not originate as the nuclear wall itself; there are cytologists who would perhaps deny that a nucleus has a wall of a more definite nature than a simple film separating one cell phase from another. It is possible that a new wall is produced in the cytoplasm closely surrounding the nucleus. The stages shown in Figure 6, K to M, however, indicate fairly conclusively that the nuclear membrane becomes the wall of the ascus.[6]

Seeking among other fungi for a parallel to the process of free cell division in Ceratostomella, one finds what at first promises to be a close approach in *Monascus purpureus* Went, as described by Ikeno (*18*) in 1903. According to Ikeno, there occurs what is essentially a fragmentation of the ascogonium, or the aggregation of cytoplasm about certain nuclei, so that numerous isolated cells or "Cytoplasmaballen" are formed. These isolated cells undergo a further fission and ultimately form "sporangia" or asci of six to eight spores. Ikeno, supposing that the fungus was not a true Ascomycete, regarded the free cells as "spore mother cells."

A further search of the literature on Monascus shows a remarkable series of contradictions and reinterpretations (*1*, *19*, *25*), culminating

[6] In respect to the multiplication of asci by the division or fragmentation of naked and unattached cells the observations reported here are supported by those of Mittmann (*21*). Apparently due to a misinterpretation of the nucleus, Mittmann does not reach the same conclusion in regard to the origin of the ascus wall. The structure which she identifies as a nucleus is believed to be only a nucleolus, as will be shown clearly by comparison of her drawings with Figure 6 of this paper.

in the most recent new "interpretation" by Young (*29*) on *Monascus ruber* Tiegh. This paper gives an entirely new aspect to the Cytoplasmaballen of Ikeno, the author supposing that they are in fact ascospores in process of formation. Young does not account satisfactorily for the final multiplicity of asci, and her interpretation is not entirely convincing to one familiar with the sequences in the development of these organs. There is still a strong possibility that the earlier deductions of Ikeno may prove to be essentially correct.

One fact stands out conspicuously in most of the work done on the morphology of fungi of this type. This is the obscurity surrounding the early stages in the multiplication of asci. For most of the Sphaeriales these stages have been passed over lightly, it being supposed that ascogenous hyphae are formed comparable, for example, to those in Pyronema (*11*). It is becoming increasingly evident that the study of ascomycetous fungi should include a more careful examination of early stages of development and that classification should not follow too closely the characters of the mature fruiting body. It is possible that in the early proliferations of the ascogonium will be found characters of much value in indicating relationships among Pyrenomycetes.

Certain features in the life history of *Ceratostomella fimbriata* discussed in this paper may be regarded as unique among true Ascomycetes. They perhaps indicate some degree of affinity with the Hemiascomycetes. That these same features are essentially true also for *C. adiposum* has been borne out by limited observations on the latter species. It appears probable also that corresponding stages in the development of *C. piceae* as reported by Varitchak (*26*) (judging from his figures rather than his statements) differ only in detail from those in *C. fimbriata* and *C. adiposum*. The process of free cell fission in the perithecium as described here might well be regarded as an adequate basis for a new order of Pyrenomycetes. The process possibly is associated with the deliquescence of asci in the perithecium, a feature that distinguishes a considerable number of the Sphaeriales, but such an association can not be assumed to exist. Therefore, if a truly natural classification of fungi is an ideal worth attaining it will require a closer scrutiny of developmental processes than has been devoted to them in the past.

SUMMARY

Asexual reproduction in *Ceratostomella fimbriata* is accomplished by two conidial forms. Both types of conidia are abstricted within the sheath of the sporophore and are known as "endoconidia."

The fungus is one of a group of Pyrenomycetes notable for the early disintegration of ascus walls.

The organism is entirely homothallic. Antheridium and oogonium are borne on the same hyphal branch. A large number of single ascospore isolations have failed to reveal any evidence of sexual strains.

The antheridium is believed to be functionless, fertilization being effected by a nucleus arising in the terminal region of the ascogone.

The subterminal cell of the perithecial stalk normally becomes binucleate before any enveloping hyphae are in place, and this binucleate condition persists up to the time of ascus formation.

There is only one nuclear fusion in the life cycle; this occurs in the young asci.

The oogonium becomes the primary ascus cell; its wall dissolves, and it lies free in the cavity of the young perithecium.

As the perithecium increases in size by new hyphal growth a digestive action enlarges the inner cavity by a dissolution of the internal cells.

Neither paraphyses nor periphyses are formed.

No ascogenous hyphae are formed. Fragmentation of the oogonium and of its daughter cells results in a multitude of naked cells embedded in a nutrient medium and entirely detached within the perithecial cavity.

These ascogenous cells may cling together in series of two to four, but they are without hyphal connection with the perithecial wall or its base.

The method of division or constriction of the ascogenous cells gives the false appearance of an ascus hook or crozier.

The two nuclei in certain of these cells fuse at an early stage and this large definitive nucleus persists for a relatively long period, after which three divisions in rapid succession result in a cell of eight nuclei and the later delimitation of eight ascospores.

The first of the three nuclear divisions is characterized by the appearance of an ascus vesicle, the wall of which appears to be the membrane of the fusion nucleus.

Subsequent nuclear divisions and the delimitation of ascospores appear to take place within this membrane. The wall of the fusion nucleus therefore becomes the wall of the ascus.

Ascus walls are dissolved early, often before the spores are quite mature, so that a mature perithecium contains numerous free ascospores that are ejected through the beak in a mucilaginous coil when the perithecium is moistened.

The process of free cell division that results in a multiplication of asci may be a more accurate measure of the phylogenetic relationship of the genus than the characters now being employed.

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VEIN SPOT OF THE PECAN CAUSED BY LEPTOTHYRIUM NERVISEDUM, N. SP.[1]

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INTRODUCTION

Vein spot, apparently an unreported disease of the pecan (*Hicoria pecan* (Marsh.) Britton), appears to be one of the principal foliage diseases of pecans in the southwestern part of the United States. Demaree [3] collected the vein-spot fungus near Austin, Tex., in 1920, and at Ferriday, La., in 1929, and the writer has collected it on both native and cultivated pecans in Louisiana, Mississippi, Arkansas, and Texas. The disease has not been reported from the southeastern pecan belt, which includes Alabama, Georgia, Florida, South Carolina, and North Carolina.

THE DISEASE

In the vicinity of Shreveport, La., vein spot first appears in May or June on the foliage of vigorous pecan trees, and in some orchards it causes the leaves of certain varieties to shed prematurely. The disease may attack the rachis, the petiole, or the veins, but the entrance of the fungus is always confined to the vascular system of the leaf. Infection may take place at or near the end of the midrib of a leaflet and extend down the midrib to its base (fig. 1), producing a narrow black necrotic streak. The discoloration of the leaf tissues seldom extends more than 2 mm or 3 mm on either side of the midrib. The infection may also extend from the main vascular bundle that runs lengthwise to the smaller lateral ones. When numerous small lateral bundles are killed the leaf tissue between them dies, forming large dead areas that may later involve the entire leaflet, causing it to drop. The fungus may attack either the terminal or the basal portion of the rachis and gradually extend to the opposite end (fig. 2), causing the leaflets to fall one at a time until finally the rachis, devoid of leaflets, is left hanging on the tree. In other instances, when either primary or secondary veins become infected, the fungus does not spread extensively, but causes a small oval-to-circular black spot. A leaflet may contain 8 or 10 of these black spots, which are sometimes not over 2 mm in diameter. The vein divides such spots into two almost equal parts. In some instances the fungus attacks the midrib of a leaflet and finally causes it to shed without interfering with the rest of the leaf (fig. 4), whereas in other instances it seems to attack the rachis, midrib, and vein simultaneously (figs. 1–3). In serious cases,

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[3] This information was obtained by the writer through correspondence with J. B. Demaree, pathologist, Division of Horticultural Plants and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.

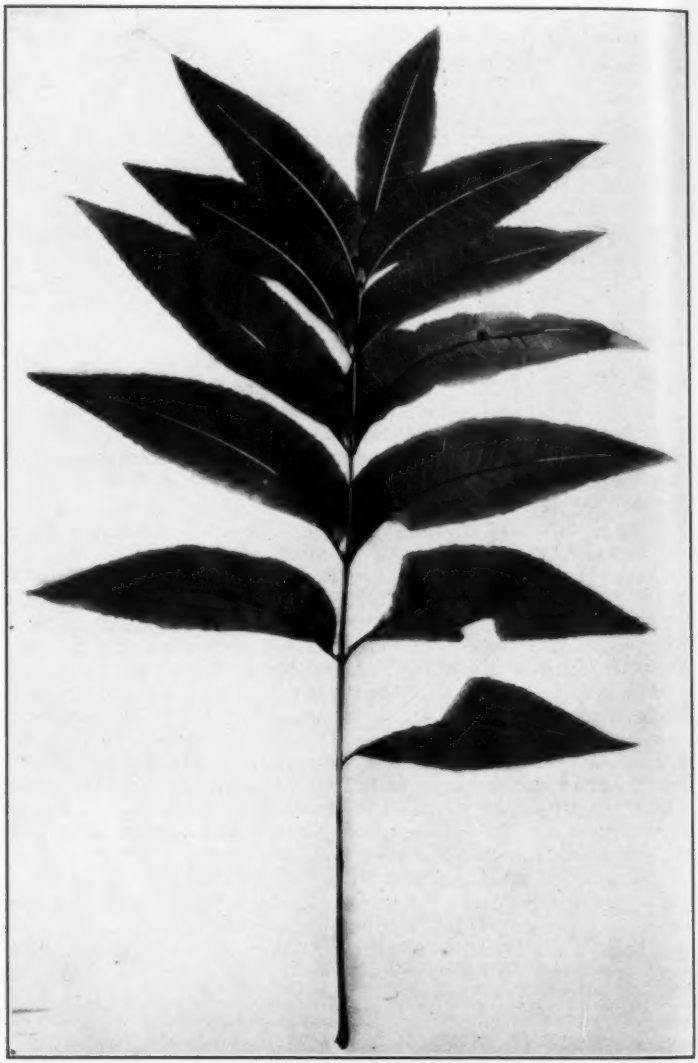

FIGURE 1.—Characteristic appearance of a pecan leaf attacked by the vein-spot fungus. The discoloration is confined strictly to the midribs and veins of the leaflets and to the petiole

such as occurred in the vicinity of Austin, Tex., the entire vascular system of many leaves is attacked, and severe defoliation results.

The spots which appear on the lower or ventral side of the leaves and four or five days later on the dorsal surface are yellowish at first and later brown, finally turning dark brown or black with yellowish margins. In the later stages of the disease the spots on both surfaces of the leaves have about the same appearance, except when fruiting

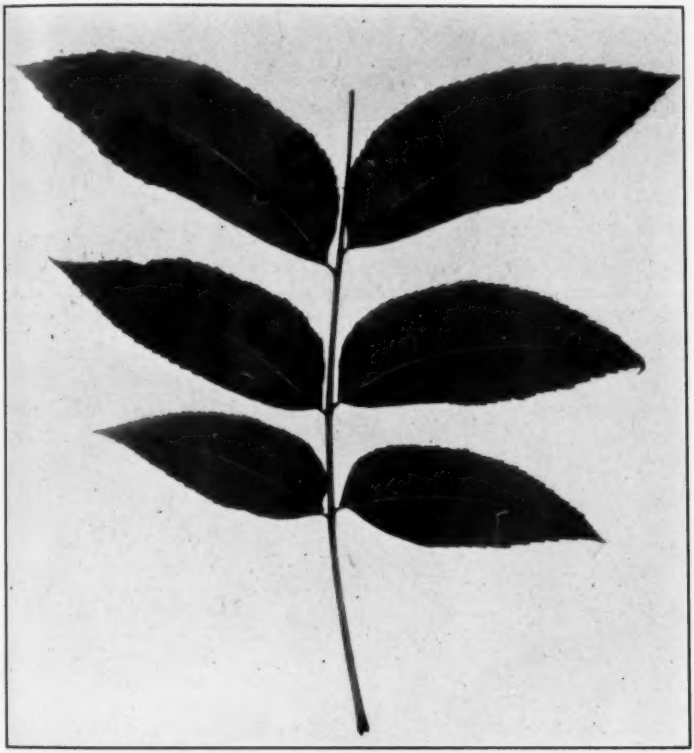

FIGURE 2.—Pecan leaf severely injured by the vein-spot fungus. The terminal leaflets have fallen and the petiole is discolored

bodies of the fungus are present. These fruiting bodies nearly always occur on the lower leaf surface and are usually confined to that portion of the lesion involving the vascular system.

Because of its striking similarity to pecan scab (*Cladosporium effusum* (Wint.) Demaree), vein spot has perhaps been confused with scab by growers and phytopathologists. Since scab spots no longer sporulating are black and are about the same shape and size as lesions of the vein-spot disease and since scab spots also appear on the leaf veins, it is difficult by casual examination to differentiate between scab and vein-spot lesions.

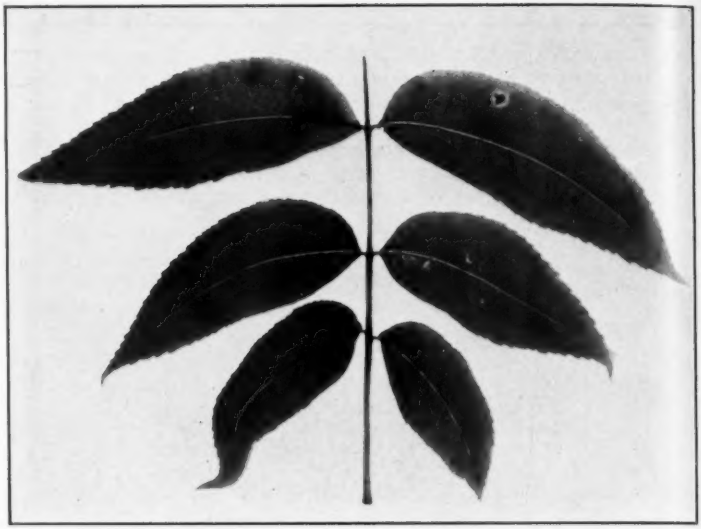

FIGURE 3.—Basal and upper leaflets of pecan leaf, showing characteristic lesions of the vein-spot disease. The terminal leaflets have fallen

FIGURE 4.—Terminal leaflets attacked by the vein-spot fungus. Note the discoloration of the petiole and leaflet. The remainder of this leaf was normal

THE CAUSAL FUNGUS

The fungus causing vein spot does not begin fruiting until late in the summer or in the fall. In the vicinity of Shreveport, La., a search for fruiting bodies on diseased leaves was made from June to September, 1930, but none was found earlier than about the first of September; they were not very abundant until about the middle of October.

MORPHOLOGY

Just before the formation of fruiting bodies, which are considered as dimidiate pycnidia, the diseased areas on the underside of the leaf take on a grayish color. The fruiting bodies usually appear in these areas, either singly or in groups, and are difficult to see without some magnification. Usually they are intraepidermal or subepidermal (fig. 5), but occasionally they are found in the spongy parenchyma (fig. 6), and vary from 35μ to 95μ in diameter. They are embedded in the host tissues, apparently replacing the epidermal wall bordering on the spongy parenchyma and gradually destroying part of the latter. The covering of the fruiting body is irregular in outline and is composed of the somewhat thickened and blackened cuticle and epidermis. (Fig. 7.) This covering is occasionally torn in microtome sections, and several layers of tissue are found to be present. The coloring is probably due to some substance secreted by the fungus. Fungous tissue is no doubt present, but it is somewhat difficult to demonstrate it in the pycnidium. The base is made up of stromatic cells that penetrate the spongy parenchyma. In some instances layers four to five cells deep are formed.

TECHNICAL DESCRIPTION

The fungus herein described is a new species with the following diagnosis:

Leptothyrium nervisedum, n. sp.

Pycnidiis hypophyllis, depressis-pulvinatis, 35μ–95μ latis; conidiophoris simplicibus, septatis, rectis vel leniter curvatis, 14μ–18μ longis, 2u–4μ latis; conidiis irregularibus interdum curvatis, oblongis-ovatis, hyalinis, continuis, 8μ–13μ longis, 2μ–3μ latis.

In foliis *Hicoriae pecan* (Marsh.) Britton, Louisiana, Mississippi, Arkansas, et Texas.

Pycnidia hypophyllous, depressed pulvinate, 35μ–95μ wide, conidiophores simple, septate, straight or slightly curved, 14μ–18μ by 2μ–4μ; conidia irregular, sometimes curved, oblong to ovate, hyaline, nonseptate, 8μ–13μ by 2μ–3μ.

Lesions occur only on the vascular system of foliage of *Hicoria pecan* (Marsh.) Britton. The disease is found in Louisiana, Mississippi, Arkansas, and Texas.

When germination begins, a septum is usually formed dividing the conidium into almost even parts. Two germ tubes may come out from the conidium, one from each end (fig. 7), or the tubes may come out at either side.

PHYSIOLOGY

The fungus causing the vein-spot disease of pecans was first cultured from mycelium early in the season. The cultures were made by placing the diseased host tissues in Petri dishes containing either cornmeal or pecan-leaf agar. Growth from the mycelium was very slow, the fungus requiring from two to three weeks to become macroscopic. Fruiting bodies were produced on the leaves in late summer, but the spores did not germinate until the latter part of October. When

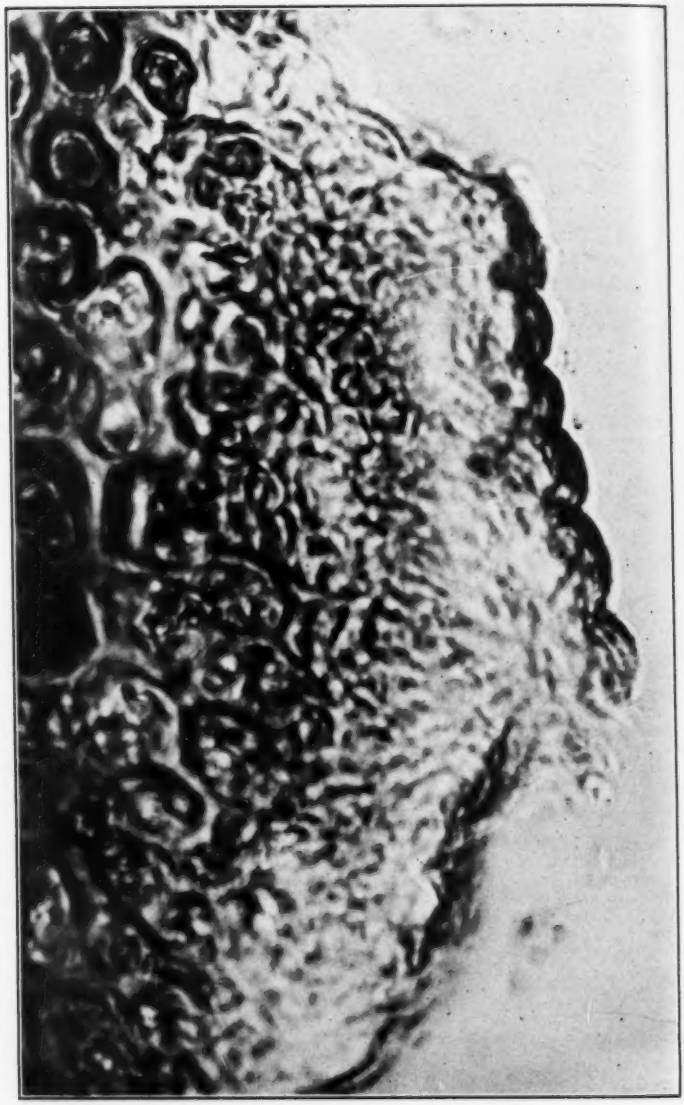

FIGURE 5.—Photomicrograph showing fruiting body of the vein-spot fungus produced above the spongy parenchyma of a pecan leaflet. The epidermal cells have been completely destroyed. × 1,700

placed on corn-meal or pecan-leaf agar, the spores sent out germ tubes from either end and sometimes from both ends. (Fig. 7.) Growth from the spores was likewise very slow at first, 15 days at 25° C. elapsing before monospore cultures became macroscopic.

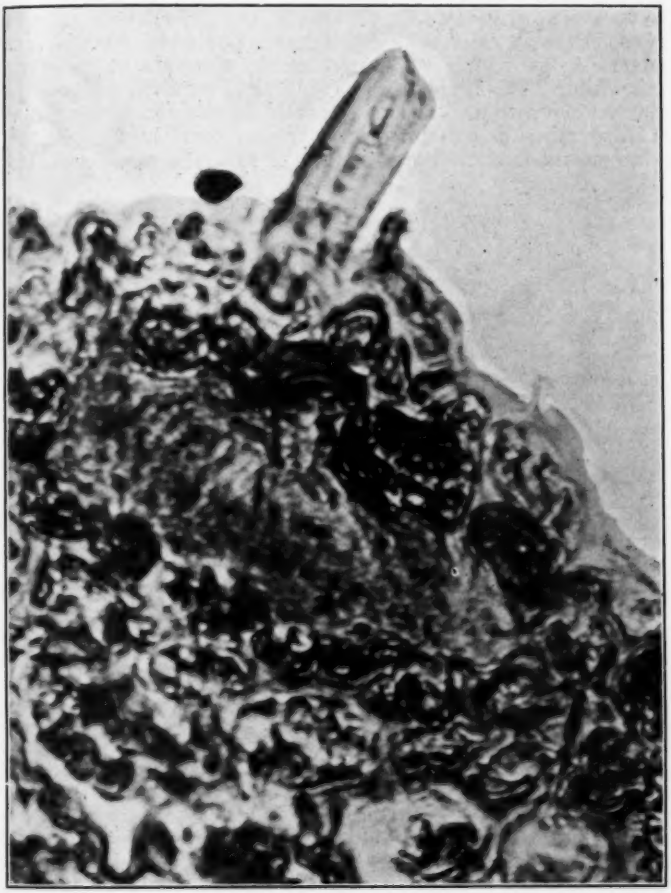

FIGURE 6.—Photomicrograph showing fruiting body of the vein-spot fungus produced in the spongy parenchyma of a pecan leaflet. The plant hair extends from the epidermal cells directly above the fruiting body. × 1,700

The fungus was grown on a variety of media, including nutrient agars, pecan-leaf and hickory-leaf agars, and vegetable plugs. A few pycnidia and spores were produced on corn-meal and on pecan-leaf agar, but the best growth, 9 cm in diameter, was made on potato plugs. In each instance the fungous growth consisted of a dark-brown to black tough stroma, mostly submerged and covered with short aerial hyphae.

TEMPERATURE AND pH STUDIES

The vein-spot fungus was grown in an incubator[4] at constant temperatures ranging from 15° to 36° C. Poor growth occurred at 15°, whereas at the optimum temperature (26°) the fungus covered an area 7 cm in diameter. In all the compartments, except the one held at 36° where no growth occurred, the fungous growth was mostly submerged, brown in color, and covered with white or light-brown aerial hyphae. A few spores were produced in pycnidia at the optimum temperature. At all temperatures the fungus secreted a pigment that discolored the media. The color varied from light to dark brown, depending upon the amount of growth made by the fungus. When the fungus made optimum growth, the media became dark brown.

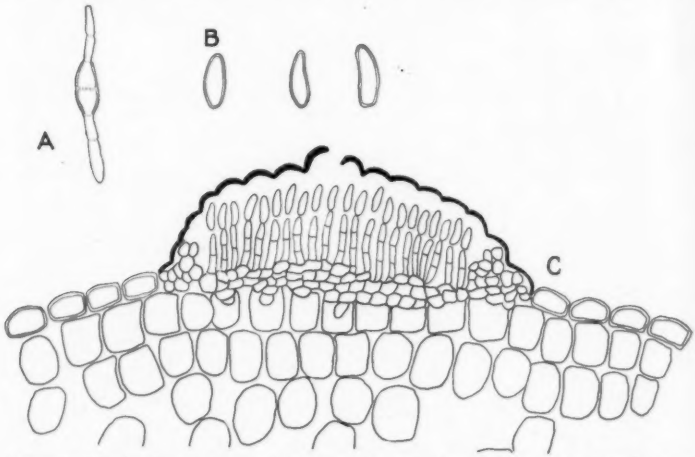

FIGURE 7.—The vein-spot fungus. A, Germinating conidium; drawing made after 30 hours. × 500. B, Representative conidia. × 500. C, Cross section of a leaf with the acervulus on the midrib. × 250

In order to study the influence of the hydrogen-ion content of the medium upon the behavior of the fungus, corn-meal agar was used in lots differing in acidity by 0.3 pH over a range of pH 4.0 to pH 8.6. The optimum growth (6.5 cm in diameter) was made at pH 5.9, whereas poor growth was made at pH 4.0 and pH 8.6. At all the degrees of hydrogen-ion concentration used, the appearance of the fungus was characteristic. The stroma was black, tough, mostly submerged, and covered with light to dark, short, aerial hyphae. The medium was also discolored, the discoloration depending upon the growth of the fungus, being dark brown at pH 5.9, the optimum growth, and lighter at pH 4.0 and pH 8.6. A few spores were produced in pycnidia at pH 6.6 and pH 6.9. Some of the spores were distorted, but most of them were normal.

PATHOGENICITY

Pecan leaves of the Van Deman variety were inoculated with a heavy suspension of conidia taken from the diseased Van Deman

[4] The incubator consisted of 8 compartments each large enough to hold 2 dozen test tubes and 1 thermometer. The compartments were connected, with an ice-box at one end and a light bulb at the other. The incubator was so constructed and insulated that the temperature did not fluctuate more than 2°.

leaves collected in 1930. The inoculations were made as follows: Conidia were taken from diseased leaves and placed in a watch glass containing distilled water. Some were then placed on the leaves on or near the midrib or veins; others were placed between the veins. A little crayon was added to the inoculum to mark the inoculations. The leaves were then placed in glassine bags and kept there for 48 hours. The results are shown in Table 1.

TABLE 1.—*Results of inoculating leaves of the Van Deman variety of pecan with conidia of the vein-spot fungus*

Series No.*	Inoculations		Infections		Remarks
	Date	Number	Date of reading	Number	
1	Apr. 22	25	May 15	11	Spots, 1–3 mm long.
2	May 1	50	May 20	34	Do.
3	May 5	50	May 24	29	Do.
4	Aug. 9	25	Sept. 1	3	

* In series 1, 2, and 3, the inoculations were made on the veins; in series 4, the inoculations were made between the veins.

Uninoculated leaves held as checks remained free from the disease. The incubation period for the fungus appears to be between two and three weeks. In all instances infection took place on the vein or midrib, regardless of where the inoculations were made. In series 4 the infections took place on the veinlets, to which the spores were probably carried by insects or water from the place of inoculation.

In an effort to find the perfect stage of the fungus, infected leaves were collected in October, 1930, and placed outdoors in wire cages to overwinter. The leaves were examined at intervals of four weeks in order to ascertain whether any change in the life history was taking place; but no change was observed, and the conidia were found to be viable on May 1 of the following year. It is evident, therefore, that the fungus passes the winter in the conidial stage.

VARIETAL SUSCEPTIBILITY

Although vein spot has been found on both native and cultivated pecans, it is much more common on the cultivated varieties. In Louisiana the Van Deman variety is the most susceptible and the Frotscher variety is the next most susceptible; other varieties are attacked to about the same degree, depending somewhat on the locality. The writer is not familiar with many of the Texas varieties; the Kincaid variety, however, appeared to be attacked more severely than any of the others observed during the fall of 1931. Since both the Van Deman and the Frotscher varieties scab badly in southern Louisiana, it is sometimes difficult to distinguish between old scab lesions and vein-spot lesions. Scab causes more serious damage to the nuts than to the foliage; it is rarely serious enough to cause defoliation by itself, but probably acts in conjunction with other foliage diseases, especially vein spot, in defoliating the trees.

In the vicinity of Shreveport, La., the Van Deman is the only cultivated variety of pecan that has been attacked to any great extent, but since attacks by the fungus seem to be increasing on other varieties, vein spot may prove to be a serious disease within the next few years. There is some evidence that various strains of the vein-spot fungus exist. Other varieties of pecan which have been free from this disease but which grow near trees of the Van Deman variety

that have been prematurely defoliated for several years are also becoming diseased by the vein-spot fungus. It may be that the fungus is gradually adapting itself to other varieties. For example, in one orchard where Schley trees are growing near Van Deman trees that have been prematurely defoliated for the past two years, the writer was unable to find any signs of the disease on the Schley during the season of 1930, but in 1931 a few of the Schley leaflets were infected. There are instances of other varieties that have become infected in a similar manner.

Since the disease is present near the Gulf coast in Louisiana and Mississippi, where the annual rainfall is about 60 inches, and is also present in the central part of Texas, where the annual rainfall is only about 25 inches, it would seem that humidity is a negligible factor in its development. Probably vein spot attacked the native trees of Texas first and is now spreading eastward.

CONTROL

Although no experiments have been conducted with the object of controlling vein spot, the following observations have been made in experiments conducted for the control of pecan scab. In orchards where the growers dusted for control of scab on the Van Deman variety in 1929, using 4 applications of 20–80 monohydrated copper sulphate and lime dust, the vein-spot fungus was held in check, whereas on trees that were not dusted this fungus caused defoliation by September 15. During the season of 1931, in experiments conducted for scab control on the Frotscher variety in southern Louisiana, 4 applications of a dust composed of copper sulphate monohydrate (20 per cent) and hydrated lime (80 per cent), alternating with 3 applications of Bordeaux mixture composed of 3 pounds of copper sulphate (bluestone) and 4 pounds of lime in 50 gallons of water, resulted in about 90 per cent vein-spot control. The check trees were defoliated by vein spot, together with *Cercospora fusca* F. V. Rand. In each of the experiments just described, the first application of dust or spray was made about the middle of May, and the last was made about August 1.

SUMMARY

Vein spot, a foliage disease of the pecan (*Hicoria pecan* (Marsh.) Britton) caused by *Leptothyrium nervisedum*, here described as new, has been collected by the writer in Louisiana, Arkansas, Mississippi, and Texas.

The fungus attacks the vascular system of the leaf, thereby suggesting the name "vein spot." Heavy infection results in severe injury to the foliage or in premature defoliation.

A varietal susceptibility to the fungus appears to exist, especially in Louisiana. The Van Deman is the most susceptible variety, and the Frotscher is the next most susceptible.

Since vein spot is found both in regions of high and of low rainfall, humidity is not considered to be a factor in the development of the disease.

No experiments for the control of vein spot have been conducted. However, very good results in the control of this disease were observed in plots where experiments were in progress for controlling scab by spraying three times with 3–4–50 Bordeaux mixture, and alternating with four 20–80 monohydrated copper sulphate and lime dust treatments.

PLASTER MOLDS OCCURRING IN BEDS OF THE CULTIVATED MUSHROOM[1]

By VERA K. CHARLES, *Associate Pathologist*, and EDMUND B. LAMBERT, *Associate Pathologist, Division of Mycology and Disease Survey, Bureau of Plant Industry, United States Department of Agriculture*[2]

INTRODUCTION

Among the numerous fungi that may be found in the compost of mushroom beds in the United States, two are often conspicuous. One develops over the surface of the manure in patches that are white in the early stages of growth, turning cinnamon-brown after a few days. The other fungus grows deep into the beds, forming a white powdery mass that may become a pinkish gray but never brown. These molds are responsible for considerable economic loss, and most experienced growers are familiar with their gross appearance. A great deal of confusion, however, has arisen from the use of different common names by different growers and from the fact that the relation of these fungi to described species has not been clearly defined. In an attempt to clear up this confusion the writers have isolated and critically examined cultures of both fungi from several localities. The present paper reports data on their distribution, variability, and probable relation to species described in foreign countries. In the following discussion the subsurface mold will be designated "white plaster mold" and the surface mold "brown plaster mold".

WHITE PLASTER MOLD

White plaster mold is the more injurious of the two fungi under consideration. It penetrates deeply into the beds and gives the compost the appearance of having been dusted with flour. It greatly retards or completely inhibits the growth of mushroom spawn, so that its presence in the beds frequently results in the total failure of the crop.

The occurrence of plaster mold in the United States was first brought to the attention of the Department of Agriculture in 1897, when material was received from San Rafael, Calif., with the information that the spawn had made a certain amount of progress but had suddenly been checked. The second report of the occurrence of this disease in the United States was received in 1920, this report also coming from California. Two years later the same grower reported that a loss of over $100,000 had been suffered by California growers, and his personal loss was estimated at from $20,000 to

[1] Received for publication Feb. 23, 1933; issued July, 1933.
[2] Acknowledgment is made of a culture of white plaster mold from Dr. Louise Dosdall, University of Minnesota, and of two cultures from Dr. W. M. Ware, of England, in 1930.

$30,000 during a period of 4 years. Since then the writers have observed similar failures and have identified and isolated the fungus from collections made in Pennsylvania, Minnesota, California, Idaho, Illinois, and New York.

White plaster mold is also a serious pest in France, Italy, and England. Detailed accounts of extreme losses from this fungus are given by Costantin and Matruchot (*5*) [3] for France, and Cuboni and Megliola (*6*) for Italy. Recently Ware (*14, p. 19–20*) in England also mentioned it as a competitive invader in mushroom beds.

IDENTITY

The various collections of white plaster mold made by the writers in four different States and from many different beds agree very well with the original description of *Monilia fimicola* by Costantin and Matruchot (*5, p. 292*), who found it causing a disease of mushroom spawn in 1894. Because of the plastèrlike character of this fungus they referred to it as "le plâtre".[4] Their description is as follows:

> Mycelium colorless, septate, little branched, 2μ–5μ in diameter. In pure culture the sporiferous filaments are first in groups of 4 or 5, and the groups are separated from each other by a long portion of sterile mycelium.

The development is described as follows:

> The sporiferous filament is at first simple and fruits at its extremity, soon branching irregularly, each branch producing a chain of many spores. The fruiting filaments vary from 50μ to 100μ, are cylindrical at the base and the same size as the mycelium, diminishing gradually toward the extremity.

Unfortunately, in this description the size and shape of the spores were omitted. However, the shape of the spores and their size in relation to the mycelium are shown clearly in the drawings (*5, pl. 13, figs. 9–13*). In 1906 Saccardo (*11, p. 503*) described the spores as elliptical with obtuse ends and 6.5μ–8μ by 4.5μ–5.3μ in size. The inference is that these measurements were estimated from the magnification of the drawings. They agree with the measurements of spores in the writers' cultures. Figure 1 shows spores from a culture of *Monilia fimicola*. The U-shaped dichotomous branching of the sporophores as shown in the drawings of Costantin and Matruchot was also found to be quite characteristic of the writers' cultures. Figure 2 shows U-shaped branching of the mycelium. Figure 3 is from a culture of *M. fimicola* showing mycelium and spores.

In 1903 the Italian investigators Cuboni and Megliola (*6*) published an account of this disease in Italy. Their observations led them to the conclusion that the Italian fungus was identical with *Monilia fimicola;* but because the mycelium was limited to the substrata, and because the spores were extremely small, they referred it to the genus *Oospora*, designating it *Oospora fimicola* (Cost. and Matr.) Cub. and Megl. The two descriptions of this fungus by the French and the Italian authors, respectively, are very similar, the main difference being in the shape of the spores. Cuboni and Megliola describe them as globose, whereas those in the drawings of Costantin and Matruchot (*5*) are ovoid. In the course of this study the writers

[3] Reference is made by number (italic) to Literature Cited, p. 1097.

[4] It should be noted here that Costantin (*4*) first applied the name "plâtre" to a very different fungus, *Verticilliopsis infestans*, but later referred to *Verticilliopsis* as the "faux plâtre" of the gardeners.

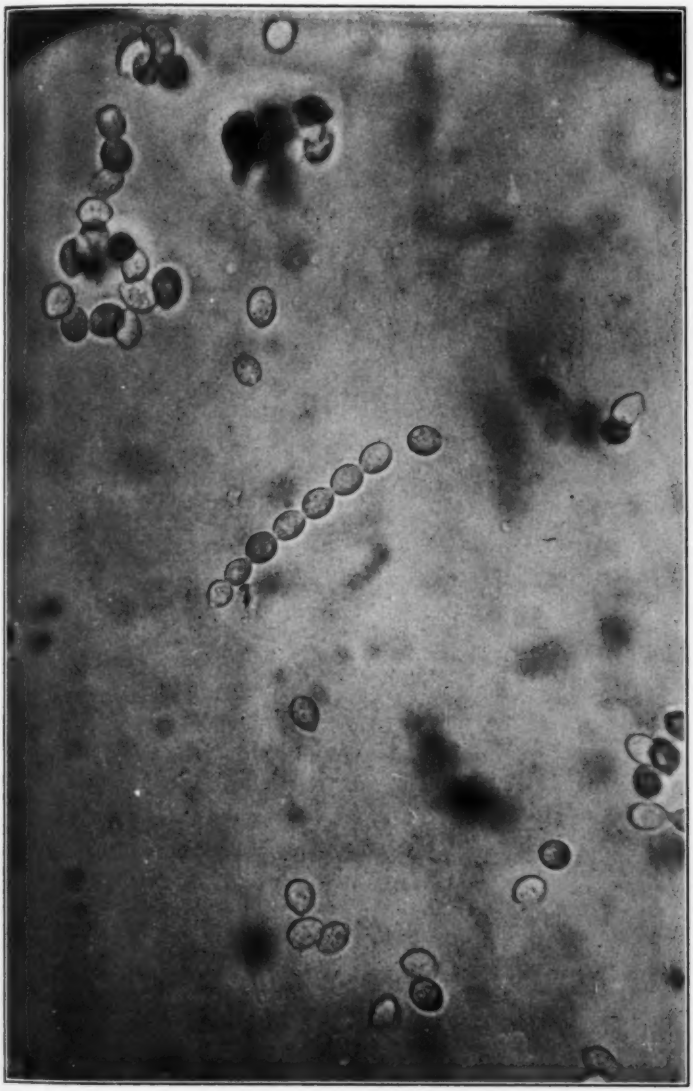

FIGURE 1.—Spores from culture of *Monilia fimicola*. × 800

examined a specimen from Cuboni distributed by D. Saccardo[5] and found only a few globose spores, the majority being oval. It is noteworthy in this connection that the spores in the Cuboni specimen also correspond in size and shape with the spores from the writers' cultures. Whether this species is to be considered as belonging to the genus Monilia or to the genus Oospora is largely a matter of interpretation. Since sterile hyphae are plentiful on ordinary media, the writers prefer the older name *Monilia fimicola* Cost. and Matr.

FIGURE 2.—U-shaped branching of the mycelium of *Monilia fimicola*. × 380

In 1930 W. M. Ware sent the writers two cultures of *Monilia fimicola* which he had collected in England in 1929. One was isolated from a sample of dung from a mushroom bed at Bradford-on-Avon, Wiltshire, and the other was from a fragment of brick spawn at Lewes, Sussex. Both of these cultures were similar in shape and size of spores and general cultural characters to those isolated by the writers in the United States.

[5] Mycotheca italica Mucedinaceae, 1572, *Oospora fimicola* (Cost. et Matr.) Cub. et Megliola, Albano (Roma)—in palea putri et in fimo equino—valde noxia mycelio Agarici campestris—November 1903, Prof. G. Cuboni.

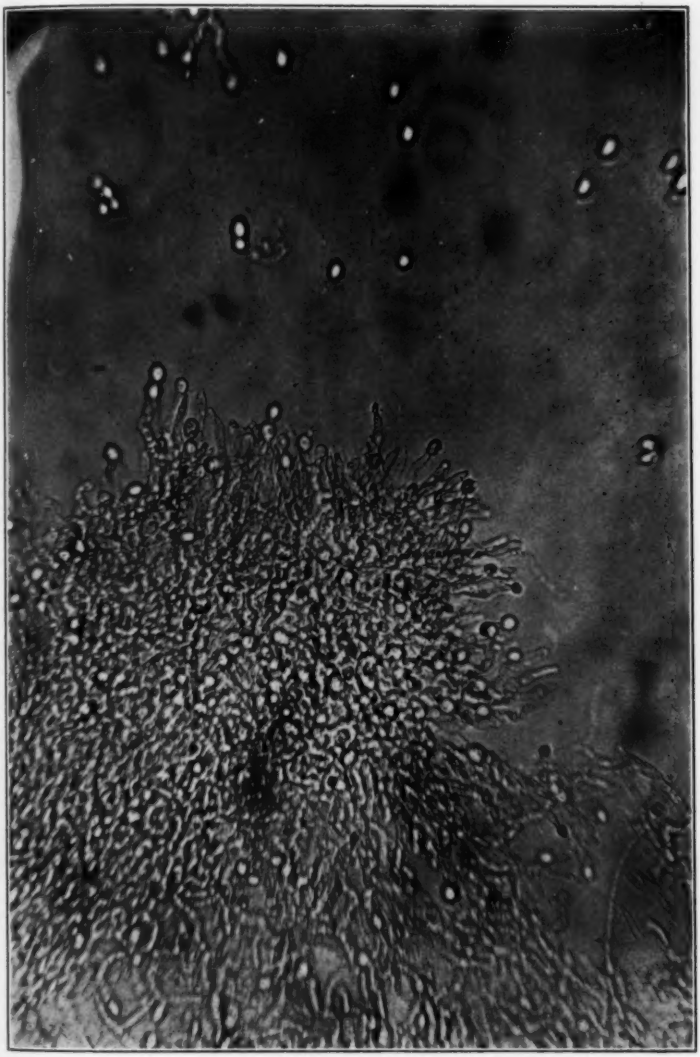

FIGURE 3.—Culture of *Monilia fimicola* showing mycelium and spores. × 380

PHYSIOLOGIC SPECIALIZATION AND MUTATION

Despite the rather constant morphology of spores and sporophores in cultures isolated from different collections of *Monilia fimicola*, there were often distinct differences in the cultural characters of strains originating from different collections when grown under identical conditions. In some cases the pale pinkish-buff color typical of the fungus mat and the coloring of the medium were entirely absent. These differences were especially noticeable when the cultures were grown in triplicate Erlenmeyer flasks on potato-dextrose agar. In the writers' opinion the differences were sufficient in four cases to indicate distinct physiologic forms, as this term is used by Stakman and his coworkers (*2*, *12*, *13*).

It is also of interest to note that sectors arose in several of the cultures when grown in flasks. These sectors were oftentimes quite

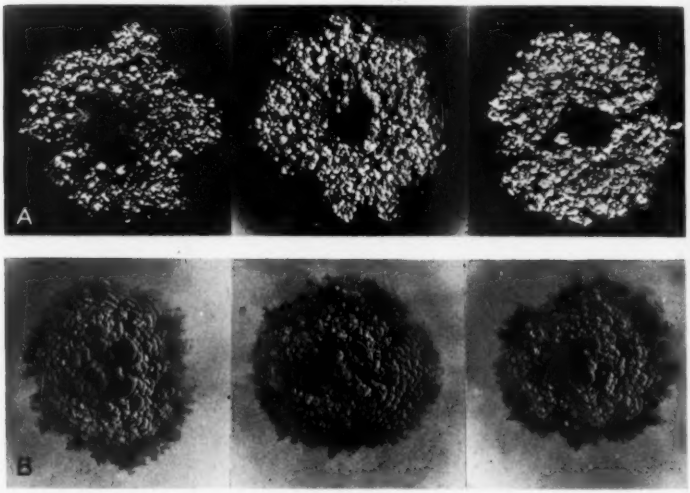

FIGURE 4.—Physiologic forms and sectoring in *Monilia fimicola:* A, Cultures of strain from California; B, from Idaho, middle culture of latter showing sector emerging. × ⅗.

distinct from the parent cultures. For example, some of the sectors were ivory-yellow and waxy, while the fungus mat of the parent cultures was pale pinkish-buff and diffuse powdery. Subcultures from these sectors had the same general cultural characters as the sectors. In monosporous cultures sectors arose which were as distinct as those appearing in mass cultures. The writers interpret these sectors as arising from mutations, as this term is used by Stakman, Christensen, et al. (*2*, *12*, *13*). The cultures shown in figure 4 are triplicate subcultures from monosporous cultures of two strains of *Monilia fimicola* grown under identical conditions on Filco potato-dextrose agar in 250-cc Erlenmeyer flasks. The strain shown in the upper row was collected in a mushroom bed near San Francisco, Calif., and that shown in the lower row was collected near Boise, Idaho. There is an evident difference in the appearance of the

fungus mat and the coloring of the medium produced by the two strains. A sector may be seen emerging from the central culture of the Idaho strain.

BROWN PLASTER MOLD

Brown plaster mold is popularly so called because of the cinnamon-brown color which characterizes it at maturity. It has been found in varying amounts in almost all commercial mushroom houses visited by the writers in the eastern part of the United States. It has been known to commercial mushroom growers for some years and was first observed by the senior writer in Pennsylvania and New York in 1923. It is typically a surface grower both when it occurs in nature and when grown artificially on culture media. Over the surface of the beds it forms patches that are usually from 6 to 15 inches in diameter. These patches, at first white and plasterlike, soon change to tan and finally to cinnamon-brown, becoming less conspicuous as they mature. In most cases similar patches may be observed under the side boards. Sometimes several patches will coalesce to form a continuous coating over the surface of the compost. When the casing soil is applied to infested beds the fungus reappears after a short delay over the surface of the casing soil. Mushroom mycelium seems to experience considerable difficulty in penetrating these spots. As a result the production of sporophores is retarded within infested areas, and the yield is noticeably reduced.

IDENTITY

If the white patches over the surface of the beds are examined with a hand lens they are seen to consist of numerous spherical bodies. Occasionally these bodies are elongated by the fusion of two. In a few instances there may be a prolongation suggestive of a neck. In size they range from 80μ to 110μ in diameter. In the early stages they consist of mostly hexagonal isodiametric cells. As they develop, the cells of the outer layer become somewhat flattened and pigmented.

Hein (*8*, *9*) isolated cultures of this fungus and found it ideal material for study of concentric rings in culture and the production of tetrakaidecahedron in pseudoparenchyma. He considers the spherical bodies sclerotia and gives excellent illustrations of their structure and cultural characters but does not attempt to identify the fungus with any known species.

The authors have found the so-called sclerotia collected from mushroom beds to be practically identical with those in a specimen of *Myriococcum praecox*[6] in the Mycological Collections of the Bureau of Plant Industry. Two other specimens of this fungus were also examined, one in the Mycological Collections[7] of the Bureau of Plant Industry and a second specimen from Denmark, determined by Rostrup. These two collections also correspond with the writers' collections. The species was described by Fries (*7*) in 1823 as the type of Myriococcum, a poorly described and little understood fungus.

The systematic position of Myriococcum is very uncertain, and by most modern authorities Myriococcum is placed among genera dubia in the Perisporiales. It was placed by Fries in the class Gasteromy-

[6] FRIES, E. SCLEROMYCETI SUECIAE, Dec. 7, No. 70.
[7] Collected by A. B. Langlois, No. 914, on decaying wood in Louisiana.

cetes, order Angiogastres, suborder Nidulariaceae, and by Corda (*3*) in the family Physarei, section Eurotiaceae. In 1842 Corda (*3*) published the results of his observations and expressed doubt as to whether or not the interior of the fungus body was chambered or hollow, but was inclined to the latter belief, as no fragment of an interior wall could be found. He stated that the spore mass filled the interior completely, and he described the spores as elongate, many sided, nearly wedge-shaped, white, and transparent. From numerous examinations it would appear that what have been called spores are in reality the inner cells which are less compressed and therefore not as uniformly hexagonal as the outer cells.

Attention may also be called to the close resemblance of *Sclerotium eurotioides* Lib. to the brown plaster mold. *S. eurotioides* was described in 1832, the description on the specimen label reading as follows: "Congestum tectum, minutissimum, globosum, leve, stramineum, demum fulvum, 100μ diameter; villo mucido demum evanescenti insidens."[8] A Libert specimen of this fungus in the Mycological Collections of the Bureau of Plant Industry appears identical in size and structure. The color is slightly lighter in the Libert material, but the difference is slight and may be due to age or the difference in the substratum. The writers are inclined to consider the spherical bodies either as abortive perithecia or the bulbils of a Hymenomycete.

DISCUSSION

Evidence has been presented which shows that the white and brown plaster molds are distinct and referable, respectively, to *Monilia fimicola* Cos. and Matr., and *Myriococcum praecox* Fries. Comparative studies of the cultural characters of eight different collections of *Monilia fimicola* from different localities in the United States and England bring out the fact that biotypes exist in this species which sometimes have quite different cultural characters under identical conditions and presumably differ likewise in their general physiology. Thus caution is necessary in generalizing from physiological data obtained for this species, such as thermal death curves, in relation to control practice. In one collection an unidentified *Monilia* was isolated which has pointed spores averaging 6μ–6.5μ by 3μ–3.5μ, but since *M. fimicola* was isolated from the same collection the significance of the unidentified fungus is problematic.

The brown plaster mold (*Myriococcum praecox*) has never been described in connection with European mushroom culture, and it is evident that the white plaster mold (*Monilia fimicola*) is identical with "le plâtre" of France and the "plaster mould" of England. For this reason the terms "brown plaster mold" and "white plaster mold" are preferred by the writers to the terms "plaster mold" (for the Myriococcum) and "flour mold" (for the Monilia), which are sometimes used (*1*). These terms are perhaps as descriptive as white and brown plaster mold under domestic conditions, but they may lead to confusion when comparisons are made between domestic and foreign conditions.

To avoid confusion of the plaster molds with other species of *Monilia* encountered in connection with mushroom culture, mention should be made of still another species of *Monilia*, one belonging to

[8] LIBERT, M. A. PLANTAE CRYPTOGAMICAE ARDUENNAE, Cent. 2 (1832), No. 138.

the *M. sitophila* group. This species has come to the attention of the writers intermittently over a long period of years as a contaminant in the manufacture of spawn. A recent French paper by Moruzi (*10*) dealing with this fungus is entitled "Sur une Maladie du Champignon de Couche Causée par un Monilia." This title would seem to be rather misleading, as the fungus was only encountered in the manufacture of spawn. Furthermore, Moruzi's paper deals with the cytology of the organism rather than with its role as an invader in mushroom beds. The writers' observations lead them to believe that *M. sitophila* cannot successfully compete with the microbial flora in well-composted manure and that it is seldom, if ever, found on the beds with the plaster molds.

SUMMARY

Serious losses are suffered by mushroom growers in the United States through the presence in mushroom beds of certain fungi popularly known as plaster molds. The most dangerous of these fungi is the white plaster mold found to be identical with "le plâtre" of France and described by Costantin and Matruchot in 1894 as *Monilia fimicola*. This species has been collected in California, Idaho, Illinois, Minnesota, Pennsylvania, and New York; and while the collections are morphologically identical, there are physiologic forms in the species, and sectors frequently appear in flask cultures. *M. fimicola* is also recognized as a serious menace to successful mushroom growing in England and Italy.

The second fungus, brown plaster mold, although commonly present in mushroom beds in the United States, has not been described in connection with mushroom culture in Europe. It is here recognized as identical with *Myriococcum praecox*, a fungus described by Fries in 1823.

Attention is also called to the occurrence of *Monilia sitophila*, which should not be confused with the plaster molds. It often appears as a troublesome contaminant in the manufacture of spawn both in the United States and Europe, but is seldom, if ever, found in composted manure with the plaster molds.

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A PHOTOMICROGRAPHIC STUDY OF GELATINIZED WHEAT STARCH[1]

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INTRODUCTION

A rigid paste of starch and water is responsible to an important degree for the physical properties of baked products made with wheat flour. Starch constitutes about 55 per cent of the weight of the starch-water fraction of bread dough and about 35 to 40 per cent of that of muffin and cake batters. The ratio of starch to its own combined water is even higher than these figures indicate because not all the water present in a baked flour mixture is free to go to starch but is associated with proteins and other constituents; nor is all the starch in bread necessarily as completely hydrated as it was in the gels studied here. Even so, such amounts of starch are relatively great as can be seen from the fact that a cooked starch paste containing only 5 per cent starch will upon cooling set to a tender gel which retains the shape of a mold.

The shapely mass produced by heating together starch and water will be called in this paper a gel, and the possibility that its internal structure may be unlike that of gels produced by particles known to be of colloidol dimensions will be ignored. Starch gels, especially those of about 5 per cent concentration, have properties in common with gels of gelatin and pectin; they appear quite homogeneous, can be cut to leave a straight-edged surface, and may show syneresis upon standing. These characteristics all play a significant rôle in starchy food preparations.

The heating of starch in the presence of water brings about changes which have been rather unanimously called "gelatinization," though different workers have interpreted the term to refer to changes with respect, in turn, to microscopic appearance of the granules, translucency of suspensions, and viscosity of fairly dilute pastes. Changes in such factors occur over a rather wide range in temperature. When gelatinization has proceeded to a certain point, a suspension will often set to the gel form already mentioned as a desirable one in many food preparations.

It was with the purpose of relating the factors of concentration, temperature of heating, and microscopic changes in the granules to the ability of a suspension to assume form as a gel that this work was undertaken. Records on hard and soft wheat starches were obtained in the form of photomicrographs showing the altered gran-

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ules at temperatures such as were found capable of producing moldable gels. Changes of starch in physical state are of interest from the standpoint of their connection with palatability and adaptability to storage and have no significant bearing, according to the literature, upon digestibility.

REVIEW OF THE LITERATURE

As a source of information concerning the microscopic characteristics of raw starch granules of almost every variety, Reichert's compilation (*21*) [2] of photomicrographs is most complete. Starches are shown under both ordinary and polarized light. Fall (*7*) has more recently published photomicrographs of a few raw specimens. As for starches which have been altered by heating in water, the literature contains descriptions but not photomicrographs. Alsberg (*1*) has in part based his explanation of the behavior of cooked pastes, an explanation now generally accepted, on the fact that microscopic observation shows that most of the granules remain whole though very much swollen. For this reason, he says an ordinary paste is a suspension, and not a colloidal solution as earlier writers have said. The viscosity of the paste, dependent upon the jostling of the swollen granules, can be much reduced by injury to the outer covering of the grain, though disruption of all granules is difficult to attain even by long grinding.

The crystallin structure of starch granules has been studied by Katz and his associates (*13*, *14*, *15*, *16*, *17*, *18*) who, with X-ray spectrograms, have found evidence of three forms—original, gelatinized, and retrograded starch. The spectra pointed to a structure within the granule which has not yet been revealed by the microscope and indicated that physicochemical changes occur in the granule during gelatinization. In microscopic appearance, gelatinized wheat-starch granules appeared to Katz (*12*) as swollen vesicles, many times their original size and showing in the center a cavity due to stronger swelling in the tangential than in the radial direction. He distinguished a first and a second degree of gelatinization encountered respectively in the changes of bread baking and in gelatinization of starch to a paste in the presence of a large volume of water. The same phenomenon of change in X-ray spectrogram was observed in both first and second degree gelatinization.

A number of investigators (*3*, *6*, *8*, *21*) have pointed out the relation between disappearance of birefringence and gelatinization temperatures of different starches. The concentration of the suspension examined by Reichert for loss of double refraction was not given, but he reported that anisotropy left wheat starch granules between 65° and 67.5° C. Alsberg (*2*) found no individual birefringent granules in bread crumb though birefringent masses could be seen. In wheat starch, the largest granules swelled and lost their anisotropy at lower temperatures than did the small ones. Alsberg and Rask (*3*) concluded as a result of viscosity measurements that gelatinization occurs over a temperature range of 25° to 30°. There was no significant change in the viscosity of 4.5 to 5.0 per cent wheat-starch pastes up to 65°, but a gradual increase occurred from 68° to 95°.

[2] Reference is made by number (italic) to Literature Cited, p. 1106.

Ripperton (*22*) devised a means of measuring the swelling power of starch in dilute suspensions and concluded from his work on potato and canna that swell and viscosity are not directly proportional. Monovalent cations increased swell and viscosity while calcium depressed them. Chapman and Buchanan (*5*) pointed out the influence of certain salts in preventing or retarding syneresis by forming a firmer gel structure. Woodruff and Nicoli (*23*) showed that large amounts of sugar destroyed the gel-forming power of 5 per cent starch suspensions. Hall (*11*) found that wheat starch heated for 1 hour at 100° C. in the presence of one-half its weight of water remained as a white powder though each individual granule had swollen. When heated under these conditions but with an equal weight of water, it became transparent, opalescent, and extremely swollen. Woodruff and Nicoli (*23*) were able to obtain a moldable gel at 90° when 5 per cent suspensions of a commercial wheat starch were handled.

Unusually great variation in the size of wheat-starch granules is a characteristic which several investigators have attempted to correlate with physical behavior. Furry (*9*) found that an average of 50 wheat-starch granules swelled to about 5 times their original size. This increase was higher than that for any other starch measured, except potato. Buchanan and Naudain (*4*) thought that superior bread-making qualities of hard-wheat flours might be correlated in some way with the high percentage of small starch grains found in hard-wheat flours as compared with soft ones. Rask and Alsberg (*20*) also found variation in the viscosity of pastes made of hard and soft wheat starches and thought that this factor might possibly be concerned with baking qualities. Grewe and Bailey (*10*), however, found no correlation between baking tests, diastatic activity or heat of imbibition, and the size of starch granules. Ripperton (*22*) said that neither size of granules of potato and canna starches nor the number of unbroken ones present could account for the differences in viscosity that he observed.

The ability of a starch paste to form a gel varies with the source of starch (*23*) and, it is believed by the authors, with the conditions of heating. Such variable factors as were studied here might easily occur in the common methods of processing used in the factory or home.

EXPERIMENTAL METHODS

PREPARATION OF WHEAT STARCH

Starch was specially prepared from flours which had been milled for another experiment-station project from single varieties of wheat. One was Fulhio, a soft red winter wheat which had been milled in a commercial soft-wheat mill. The other was a hard winter Turkey wheat milled in a hard-wheat laboratory mill. The method of separating the starch was that recommended by Alsberg and Rask except that during the washing of the starch from the gluten the starch suspension was passed through silk bolting cloth of 125 meshes per lineal inch. The starch was air-dried for use and was free from extraneous matter as shown by the following analyses: For hard-wheat starch, moisture 10.65 per cent, ash 0.09 per cent, starch (by Rask's (*19*) method) 88.92 per cent; for soft-wheat starch, moisture 10.60 per cent, ash 0.08 per cent, starch 89.39 per cent.

Starch-water suspensions containing 5, 10, and 50 per cent starch by weight were used because of the likely occurrence of such concentrations in common starchy food preparations.

METHOD OF GELATINIZING THE STARCH

One hundred grams of a combination of starch and water were mixed in 200-cc glass jars whose screw caps carried holes for a thermometer and a sampling pipette. The jars were immersed in a water bath almost to the metal cap. The suspensions were stirred with a glass rod at 15-minute intervals while they were being heated and until they reached 80° C. after which they showed no tendency to settle. The 50 per cent suspensions were stirred just before 60° after which they were too stiff for further manipulation. At specified temperatures, samples of the 5 and 10 per cent suspensions were removed by pipettes to fill small crucibles which served as molds. Samples were scooped out of the center of the 50 per cent paste with a hemispherical metal spoon. The gels were turned out onto watch crystals after 24 hours and photographed. Samples were taken for microscopic examination at the same time that they were removed for gel purposes.

A large water bath was electrically heated and stirred and was so adjusted that its temperature rose from 50° to 93° C. in 45 minutes. When the bath was already at a temperature of 93° before the jars were entered, the temperature of the suspensions reached 90° in 20 minutes. Almost continuous stirring was required with this more rapid rate of heating. A thermostatic control made it possible to hold the temperature at 90° for as long a time as desired. This large electric bath attained no higher temperature than 99.5° and permitted a maximum temperature inside the jar of only 95° to 96°.

Preliminary work had indicated that the physical character of the resulting gel depended somewhat upon the rate of heating as well as upon the final temperature reached. In view of this fact, suspensions were heated more rapidly than the electric bath permitted by immersing conical flasks containing them in a large beaker of boiling water, and still more rapidly in another case by heating the contents of the flask to boiling over a direct flame. The flask was removed as soon as the contents boiled. The temperature was found to be 99° to 100° C.

In certain instances, all cold water was used in making the 5 per cent suspension; in others, the starch for 100 g of paste was moistened with 10 cc of cold water, and the remainder of the water was added near its boiling point from a pipette. The contents were mixed by being swirled, and by the time all the hot water was added the temperature had fallen to about 70° C. In some cases, hot water was used for the first mixing even though the suspension was later to be heated in a bath whose temperature at the outset was only little above room temperature. The 10 and 50 per cent suspensions were mixed by the first method and were heated slowly in the electric bath from room temperature to the one indicated.

MICROSCOPIC TECHNIC

Samples of starch paste were removed by pipette from the jar at the desired temperatures and before the paste had solidified; one wire loop of suspension was transferred to two loops of water on a micro-

scope slide. In the case of the stiff 50 per cent suspensions, a pinhead portion taken from the center of a freshly opened lump was used for mounting. By a gentle upward and downward motion of the loop, the starch and water were mixed and spread. A cover glass was adjusted without pressure and sealed at once to the slide with a preparation of beeswax and paraffin. This kept the granules moist

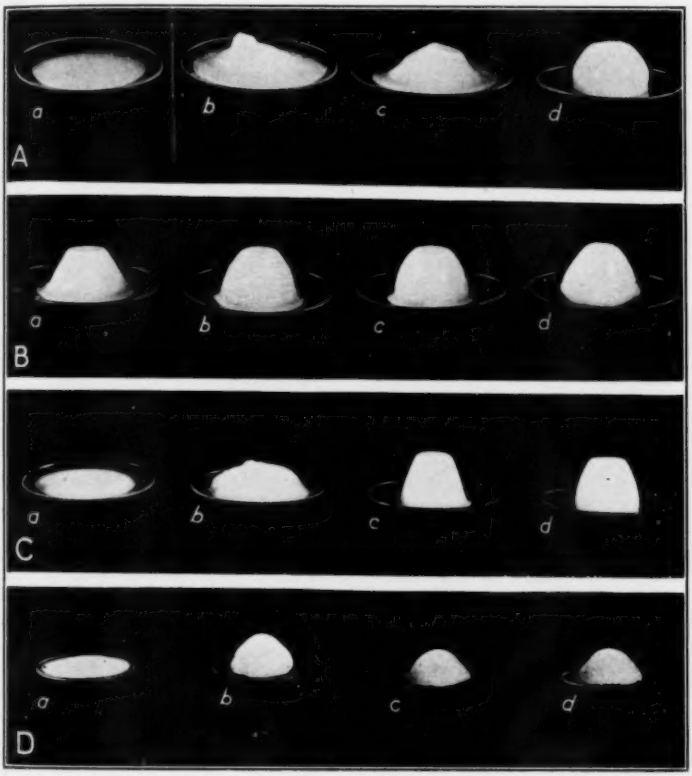


FIGURE 1.—Gels formed by hard-wheat starch: A, cold 5 per cent suspensions heated slowly from room temperature, in electric bath, *a* to 88° C., *b* to 90°, *c* to 90°, and held there 20 minutes, and *d* to 96°; B, hot 5 per cent suspensions each heated differently, *a* in electric bath from room temperature to 90°, *b* in hot electric bath to 90°, *c* in beaker of boiling water to 90°, and *d* to boiling over a flame; C, cold 10 per cent suspensions heated slowly from room temperature, in electric bath, *a* to 70°, *b* to 75°, *c* to 80°, and *d* to 95°; D, cold 50 per cent suspensions heated slowly from room temperature, in electric bath, *a* to 50°, *b* to 55°, *c* to 90°, and *d* to 95°

and prevented movement of the suspension during photographic exposure. Specimens showed no change in many hours when so sealed.

A field, representative of the whole slide, was photographed with a camera attached directly to a polarizing biological microscope carrying a 12× ocular and 30× objective. The field was photographed with ordinary light and a No. B 58 Wratten filter and with an exposure

of three minutes on a panchromatic film. Such fields as showed a considerable number of birefringent granules were also photographed with polarized light, in which case 30 minutes exposure of a nitrate film was required.

DISCUSSION OF RESULTS

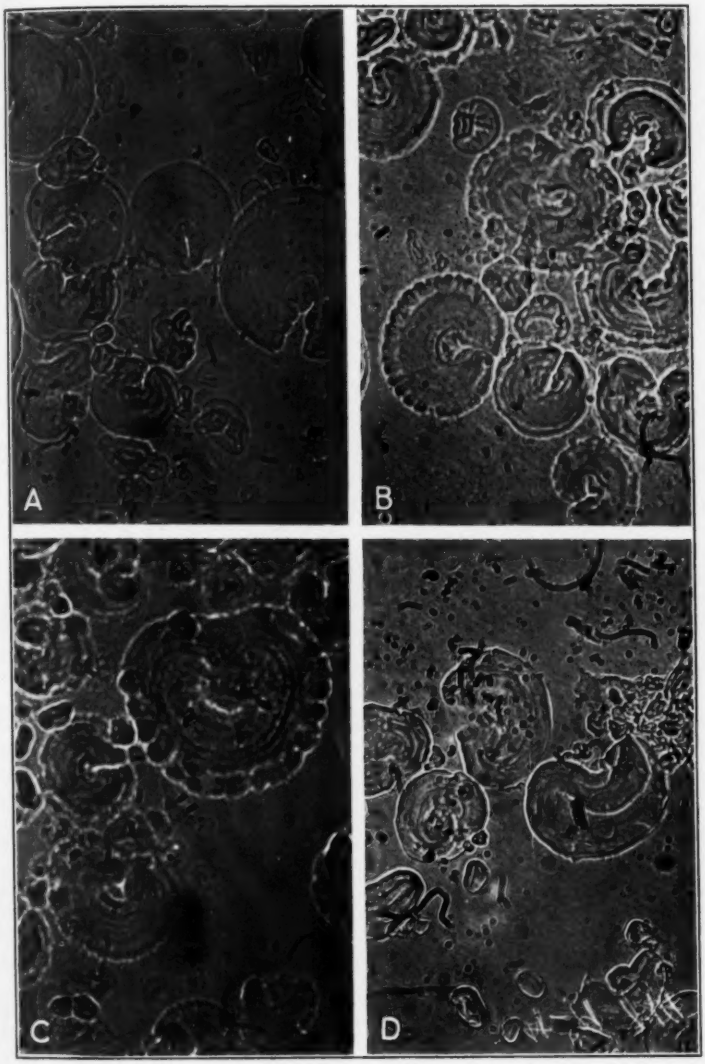
Photomicrographs showing the changes which took place as 5 per cent starch suspensions were heated from room temperature to 96° C. have been made at temperature intervals of 5° for both hard and soft wheat starches, though only a few are reproduced here. These microscopic changes were examined in conjunction with the gel-forming powers of the heated suspension. The ability to form gels was recorded photographically for suspensions heated to temperatures corresponding to those of the suspensions used in the photomicrographs. So little difference was found between hard and soft wheat starches that detailed results for each are not warranted. Hard-wheat starch is shown in the accompanying figures.

CHARACTERISTICS OF GELS

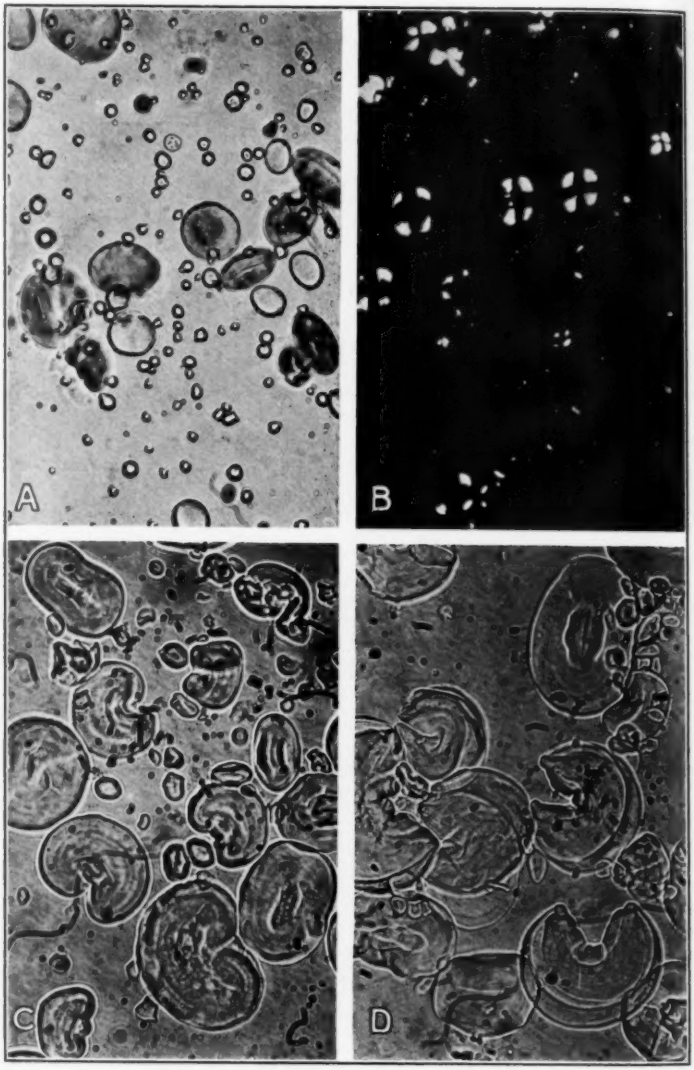
In the photographs of the gels the outline indicates whether the mass was a firm gel or but a partly formed one. In some of the 5 per cent ones the outline was clear-cut (fig. 1, B, *c*), but the gel was too weak to resist the pressure of the flat side of a spatula. One as weak as this invariably showed some leaking of water, however, as soon as it was turned out of the mold, and evidence of syneresis is plainly visible in the photograph. The firmest gels obtained rebounded after pressure and showed no syneresis after standing for three to four hours on the watch glass. The weak gels were of granular texture which made the oozing of water easily possible. The firm gels were as homogeneous, so far as the naked eye could detect, as gelatin or pectin ones. This difference in grain unfortunately does not show in the photograph nor does the difference in degree of whiteness or opalescence.

When a starch suspension of 5 per cent concentration was heated to a temperature between 95° and 100° C., a firm gel structure resulted no matter what the rate of heating was. A suspension heated slowly in the electric water bath from a cold state to 96° (fig. 1, A, *d*,) was less tough, however, than one heated quickly to boiling over a flame. (Pl. 1, B, *d*.) The temperature of 90° C. seemed to be a critical one for gelation; a gel was never obtained below this temperature (fig. 1, A, *a*), and gels sometimes failed to form even at 90° if conditions were not properly chosen; also all the gels formed at this temperature showed great weakness and syneresis. Hot suspensions brought rapidly to 90° gave the firmest gels obtainable at this temperature (fig. 1, B, *a*, *b*, *c*); slow heating gave only a suggestion of a gel (fig. 1, A, *b*), and not much improvement was made even when the heating at 90° was prolonged for 20 minutes. (Fig. 1, A, *c*.) There was a gradual improvement in gel form as temperatures were increased from 90° to 95°, though no photographs for intervening temperatures are given.

Suspensions containing 10 and 50 per cent starch (fig. 1, C and D) gelated readily at temperatures below those of the 5 per cent one, and their products were of course much tougher. At 80° C. the 10



A, Raw, 5 percent suspension in ordinary light. B, raw, 5 percent suspension in polarized light. C, 5 percent suspension heated to a temperature of 65° C. D, 5 per cent suspension heated to a temperature of 75°. × 360.



A, 5 percent suspension heated to a temperature of 90° C. B, 5 percent suspension heated to a temperature of 93°. C, 5 percent suspension heated to a temperature of 96°. D, 50 percent suspension heated to a temperature of 95°. × 360.

per cent concentration was shapely, and at 53° to 55° the 50 per cent one was. Each was white and raw in appearance at these temperatures, but they became opalescent after the temperature had risen to 95°. No syneresis was seen in either. The soft-wheat starch in the 50 per cent concentration did not become moldable until 60°.

MICROSCOPIC CHANGES

Photomicrographs of unheated granules, mounted in water as were the gelatinized ones, are shown with ordinary and polarized light in Plate 1, A and B. Occasional bacterial forms should be overlooked in these water mounted specimens. The grains remained unchanged up through a temperature of 50° C., but at 55° a slight swelling and a disappearance of the dark cross which marks birefringence had become evident in the largest granules. These changes were gradual up to the point of disappearance of birefringence which was 60° for hard, and 65° for soft wheat starch. Also at 65° (pl. 1, C), the granules had become very swollen, and a few were assuming a characteristic kidney shape which seemed to be due to a swelling of the granule around a constriction reaching to the center. This shape became more pronounced as the temperature rose, and at 93° the outer edges became beaded with what appeared to be perforations. (Pl. 1, D and 2, B.) This effect could be seen either with or without a cover glass, so it is unlikely that its cause was outward pressure upon the swollen particle. The general appearance was very similar in the two starches. In 50 per cent concentration birefringence disappeared from hard-wheat starch at 75° but did not disappear from soft-wheat starch until a temperature of 85° was reached.

The appearance assumed by granules in the gels at 90° C., which in 5 per cent strength were always weak, is shown in Plate 2, A. Water was apparently not held tightly by them so long as they retained this appearance, as evidenced by the fact that syneresis was prominent. Firm, syneresis-free gels are shown in Plate 2, C. Their grains were so swollen that they were blurred and were brought into focus with difficulty. This was not true of the 50 per cent concentration heated to 95°. Plate 2, D shows the grains were swollen but much less so, and birefringence had left the smallest ones at only 85°.

The tenacity with which the swollen granules held their water seemed to depend upon the amount of it at their command, upon the temperature to which they were finally heated, and upon the rapidity with which this temperature was reached. Where little water was available for each grain, as in the 50 per cent pastes, it was all held tightly so that no syneresis could be seen. This was true even though the microscope showed not much more swelling of the grains than had occurred in 5 per cent suspensions heated to only 65° or 70° C. Photomicrographs are not shown of the almost shapeless and indistinguishable masses characteristic of the 5 per cent pastes heated rapidly to boiling. This method, it has already been said, gave the firmest gel, most nearly free from syneresis. Why the granules absorbed the water more abundantly and more permanently at the faster rate of heating and at the higher temperature will require more work to explain.

Translucency was not a requisite of a moldable paste in the two higher concentrations studied. In the 5 per cent concentration a good

gel did not form until a temperature several degrees higher than that of the region of beginning translucency had been reached. The moldability of the 10 and 50 per cent pastes at low temperatures can probably be accounted for by the fact that many grains swelled a little as contrasted with the 5 per cent ones where fewer grains swelled much.

These results showed that the temperature at which birefringence disappeared depended on the quantity of water available to the granules. Anisotropy was gone long before the 5 per cent suspension would gel and persisted long after the 50 per cent one was a solid mass.

There was nothing in any of these observations on the physical properties of hard and soft wheat starches that would lead one to expect differences in cooking results to be due to them.

SUMMARY

Some relationships of concentration, temperature of heating, microscopic changes, and ability to form a moldable gel have been recorded in the form of photomicrographs of granules and photographs of gels given by starches of soft and hard wheats.

Photomicrographs of starch, raw and heated in 5 per cent suspension to 55°, 75°, 90°, 93°, and 96° C. are reproduced showing characteristics which have not heretofore been described in the literature.

A temperature of 90° C. seemed to be a critical one for gelation of a 5 per cent suspension. Rapid heating to this temperature gave a better-formed gel than did slow heating. Temperatures of 95° or above gave the firmest gels obtained with this concentration. Photographs show by outline the firmness of gels and suggest the comparative amounts of syneresis.

In 50 per cent concentrations, heating to 95° C. caused no greater swell than did a temperature of 65° or 70° in a 5 per cent suspension. The 50 per cent concentration became moldable at 53° to 55° even though birefringence persisted up to 75°. Anisotropy disappeared at 60° in the 5 per cent suspension.

The tenacity with which the swollen granules held their water seemed to depend on the amount of it at their command, on the temperature to which they were finally heated, and on the rapidity with which this temperature was reached. Ten and fifty per cent suspensions became moldable at temperatures below the region of appearance of translucency. This was not true of the 5 per cent suspension. Many grains swelling a little apparently accomplished somewhat the same solid effect in the high concentrations as did fewer grains swelling much in the lower one.

No significant difference was found in the gels of hard and soft wheat starches.

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MILK-ENERGY FORMULAS FOR VARIOUS BREEDS OF CATTLE[1]

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INTRODUCTION

The calorific value of milk is important as a measure of the human food value of the milk, the amount of feed required by the cow for lactation, and as a measure of lactation for the study of inheritance of milking ability.

Direct determination of the calorific value of milk has not been carried out very extensively. Overman and Sanmann (*8, 9*)[2] have reported analyses, including energy, of 212 samples without classifying them as to the breed of cow. Similar data have been reported by Möllgaard (*7*) on 60 samples from Red Danish cows and 11 samples from Jersey cows; by Kahlenberg and Voris (*6*) on 134 samples from Holstein cows; and by Savini and Gargia (*11*) on about 100 samples of market milk collected in the retail trade. All these investigators have pointed out the possibility of estimating the energy value of milk from its fat percentage. Overman and Sanmann found the correlation between fat percentage and calories per kilogram of milk to be $r=0.9814\pm0.0017$.[3] That is to say, the energy content of the whole milk produced by the cow may be estimated with a high degree of accuracy from its weight and fat percentage. The equations are given in Table 3.

Kahlenberg and Voris derived the formula, $E=183f-56.73t+556.32$, where E is calories per kilogram of milk, f is the percentage of fat and t is the percentage of solids not fat. According to this equation, at a given fat percentage increasing the solids not fat decreases the energy value, which does not seem reasonable. Also, inspection of their data suggests that the cofficient of t should be positive. For example, there are four observations at $f=3.76$ and within these four we find:

Percentage of solids not fat	7.68	8.69	8.92	8.95
Calories per kilogram of milk, determined	667	705	710	720

In this particular instance, at least, energy tends to increase with the solids not fat.

The writers have taken the trouble to recompute the formula from the published analyses, without coding, by use of the method described by Brandt (*2*). The results are $R_{E.ft}=0.9930$, and $E=93.68f+44.55t-34.09$. In this equation the coefficients of f and t are both positive and substantially in the ratio of the average energy values of fat and solids not fat, while the constant factor is very small as compared with

[1] Received for publication Jan. 4, 1933; issued July, 1933.

[2] Reference is made by number (italic) to Literature Cited, p. 1120.

[3] This value was computed by coding. Use of their published data without coding gives $r=0.9874$, a substantially higher value. When dealing with such highly correlated variables it is desirable to use small class intervals.

that of Kahlenberg and Voris. By using the above example again, results from the two formulas in comparison with the determined energy values are:

Kahlenberg and Voris formula	809	751	738	737
Determined	667	705	710	720
Present formula	660	705	716	717

Clearly the present formula agrees with the observations much better than does the formula of Kahlenberg and Voris.

While the data of Overman and Sanmann were not classified as to breed directly, the fat-percentage classification itself would automatically make some breed separation. The plotted values showing the regression of energy on fat percentage seem to be entirely linear, with no evidence of any breed influence. On the other hand, the equations of Möllgaard and Kahlenberg and Voris suggest that there may be breed differences of some significance with respect to the relation between energy and fat percentage. What is needed is an extension of these direct determinations to a larger number of samples and especially to a larger population of cows in each of the several breeds. However, it seems worth while as a preliminary to a study of breed differences to see what information may be gained from the analyses of milk of the different breeds. Such information may have value also in itself.

In the present paper certain analyses from this laboratory are presented, and a comparison is made between the different breeds with respect to milk energy. The analyses used are the 212 reported by Overman and Sanmann (*8*, *9*) which include direct energy determinations, and another set of 1,999 by Overman, Sanmann, and Wright (*10*), classified by the breed of cow, and similar to the 212 except that they do not include direct energy determinations. The energy of the milk components was derived from the 212 analyses, and these component values were than applied to the 1,999 analyses in order to derive the milk energy.

ENERGY OF MILK COMPONENTS

The energy of milk obviously lies in its several components. If both the milk energy and the amounts of the several milk components are known for a series of observations, it should be possible to deduce the energy factor for each component. It should then be permissible to apply these factors to other analyses in which energy was not determined and thereby estimate the milk energy.

It may be assumed that all of the 212 analyses including energy are equally reliable and that such discrepancies as may exist among them with respect to the present point of view are due to errors or differences of a random nature; then it may be assumed that the most probable energy values of the several components may be determined by applying the principle of least squares to the whole series of observed values. For illustration, if the energy of milk resided entirely in the fat, protein, and lactose and absolutely accurate determinations of these items in three divergent samples of milk were available, as well as the accompanying milk energy, then it would be merely necessary to set up the results as three equations and solve for the energy value of fat, protein, and lactose. It is clearly not justifiable to pick 3 out of the 212 to be used in preference to any other 3. The theoreti-

cally correct procedure is to form a set of normal equations from the 212 observations and thus arrive at the proper values from all the observations. This procedure has been followed, by using the method described by Brunt (*3*) and assuming that the energy resides in (1) fat, protein, and lactose; (2) fat, protein, lactose, and ash; and (3) fat, protein, lactose, ash, and water. The results are presented in Table 1.

TABLE 1.—*Energy value of milk components in calories per gram*

Component	Values based on energy residing in—			Standard values reported by Abderhalden
	Fat, protein, lactose, ash, and water	Fat, protein, lactose, and ash	Fat, protein, and lactose	
Fat	9.312	9.434	9.253±0.065	9.23
Protein	5.358	5.161	5.853± .127	5.71
Lactose	3.987	3.480	3.693± .059	3.95
Ash	4.980±0.199	4.323±0.173		
Water	−.0356± .0059			

Table 1 shows that the values found for fat, protein, and lactose fluctuate to some extent according to the components considered in calculating the values. This suggests that the method is not adapted to the exact estimation of the energy of milk components. The standard values for fat, protein, and lactose directly determined, as given by Abderhalden (*1*), are given in the last column. The present indirectly determined values, based on fat, protein, and lactose, agree very closely with the standard values in the case of fat but poorly in the case of lactose. Lactose was determined by difference, and the poor agreement perhaps means that the errors of determination thus tend to concentrate in this portion. The good agreement of the fat values, on the other hand, may reflect a superior accuracy in the fat determination, along with the high proportion of the total milk energy represented by the fat.

It might be concluded in advance that the water has no energy value and should be left out of consideration. Statistically the value found is significant although absolutely so small that the result may be regarded as a favorable comment on the consistency of the analyses. Likewise, the ash might have been ruled out in advance as having no appreciable energy value. On analysis, the value found, while appreciable per gram, becomes of little consequence when considered in connection with the small quantity of ash present in the milk.

The question now arises as to which set of values in Table 1 may best be used in estimating milk energy from the chemical analyses. A prior one might conclude that water should certainly be excluded, and probably ash also, fat, protein, and lactose being chosen as the most rational set. We are concerned, however, in making the most accurate estimate we can of the milk energy from the milk analyses. It is not at all inconceivable that, while there is no "negative energy" to be attributed to the water of the milk as analyzed, there may nevertheless be some peculiarity associated with high-water milk which tends to make the energy determination run slightly lower. Similar reasoning may be applied to the ash. Since the analyses, without energy, which it is proposed to use, are entirely comparable with those

involved in Table 1, it seems clear that statistically the best energy estimate will be obtained by the use of all five components—that is, $E=93.12f+53.58p+39.87l+49.80a-0.356w$, where E is calories per kilogram of milk and f, p, l, a, and w are the percentages of fat, protein, lactose, ash, and water, respectively.

BREED FORMULAS

The above formula has been applied to the milk analyses, classified by breed, as published by Overman, Sanmann, and Wright (*10*). The analyses for each breed were grouped by fat percentage into classes, 2.60–2.79, 2.80–2.99, 3.00–3.19, etc., and the average of the analyses of each group determined. The formula was then applied to these averages to derive the energy values. The results are given in Table 2.

TABLE 2.—*Computed energy values and protein-energy ratios of milk from cows of various breeds and from crossbred cows, grouped by fat-percentage classes*

AYRSHIRE COWS

Fat-percentage class	Records	Fat	Protein	Lactose	Ash	Water	Energy per kilogram of milk	Protein per calorie
	Number	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Calories*	*Milligrams*
2.80 to 2.99	2	2.920	3.020	4.625	0.658	88.78	619.3	49.0
3.00 to 3.19	3	3.113	3.170	4.607	.694	88.42	646.5	49.0
3.20 to 3.39	12	3.327	3.321	4.553	.679	88.12	671.7	49.5
3.40 to 3.59	12	3.506	3.314	4.689	.655	87.84	692.3	47.9
3.60 to 3.79	27	3.706	3.325	4.673	.659	87.64	711.2	46.8
3.80 to 3.99	24	3.900	3.519	4.551	.688	87.34	736.3	47.8
4.00 to 4.19	34	4.085	3.635	4.772	.684	86.82	768.6	47.3
4.20 to 4.39	34	4.292	3.576	4.609	.686	86.85	778.3	45.9
4.40 to 4.59	20	4.498	3.720	4.821	.691	86.27	814.1	45.7
4.60 to 4.79	19	4.691	3.802	4.708	.695	86.10	832.2	45.7
4.80 to 4.99	7	4.863	3.794	4.683	.702	85.96	847.2	44.8
5.00 to 5.19	5	5.112	3.888	4.948	.703	85.35	886.3	43.9
5.20 to 5.39	5	5.314	3.976	5.026	.707	84.98	913.2	43.5
5.40 to 5.59	3	5.490	4.140	5.007	.753	84.61	940.1	44.0
5.60 to 5.79	1	5.66	4.38	4.80	.730	84.43	959.4	45.7

GUERNSEY COWS

3.60 to 3.79	3	3.720	3.727	4.963	0.702	86.89	748.0	49.8
3.80 to 3.99	7	3.914	3.360	4.684	.701	87.34	735.1	45.7
4.00 to 4.19	10	4.078	3.561	4.995	.728	86.64	775.1	45.9
4.20 to 4.39	15	4.297	3.661	4.853	.735	86.45	795.6	46.0
4.40 to 4.59	29	4.499	3.663	4.965	.717	86.15	818.2	44.8
4.60 to 4.79	38	4.689	3.757	4.958	.716	85.88	840.7	44.7
4.80 to 4.99	36	4.894	3.907	4.978	.733	85.49	869.6	44.9
5.00 to 5.19	39	5.149	3.992	4.958	.737	85.21	897.4	44.5
5.20 to 5.39	35	5.274	4.015	4.924	.745	85.05	909.4	44.1
5.40 to 5.59	24	5.485	4.199	4.927	.761	84.63	940.0	44.7
5.60 to 5.79	22	5.693	4.165	4.921	.757	84.46	957.1	43.5
5.80 to 5.99	19	5.897	4.253	4.922	.753	84.17	980.8	43.4
6.00 to 6.19	12	6.068	4.612	4.711	.770	83.78	1,008.5	45.6
6.20 to 6.39	11	6.285	4.555	4.761	.773	83.62	1,027.9	44.3
6.40 to 6.59	9	6,472	4.611	4.754	.804	83.36	1,049.6	43.9
6.60 to 6.79	5	6.662	4.770	4.740	.788	83.04	1,074.6	44.4
6.80 to 6.99	3	6.877	4.847	4.743	.829	82.70	1,101.0	44.0
7.00 to 7.19	1	7.06	4.98	4.78	.768	82.41	1,123.7	44.3
7.20 to 7.39	1	7.37	4.70	4.88	.816	82.23	1,144.0	41.1
7.60 to 7.79	2	7.650	4.790	4.605	.812	82.14	1,163.8	41.2

TABLE 2.—*Computed energy values and protein-energy ratios of milk from cows of various breeds and from crossbred cows, grouped by fat-percentage classes*—Continued

HOLSTEIN COWS

Fat-percentage class	Records	Fat	Protein	Lactose	Ash	Water	Energy per kilogram of milk	Protein per calorie
	Number	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Calories*	*Milligrams*
2.60 to 2.79	8	2.709	3.055	5.000	0.656	88.58	616.4	49.6
2.80 to 2.99	25	2.890	3.012	4.928	.666	88.51	628.6	47.9
3.00 to 3.19	40	3.104	3.169	4.846	.672	88.21	654.1	48.4
3.20 to 3.39	47	3.289	3.276	4.884	.671	87.88	678.6	48.3
3.40 to 3.59	42	3.488	3.388	4.853	.688	87.58	702.9	48.2
3.60 to 3.79	35	3.674	3.361	4.866	.672	87.43	718.5	46.8
3.80 to 3.99	29	3.874	3.502	4.863	.681	87.08	745.2	47.0
4.00 to 4.19	11	4.078	3.734	4.858	.694	86.64	777.2	48.0
4.20 to 4.39	13	4.313	3.967	4.722	.697	86.30	806.4	49.2
4.40 to 4.59	5	4.510	4.240	4.998	.736	85.51	852.6	49.7
4.60 to 4.79	4	4.700	4.075	4.757	.692	85.77	849.6	48.0
5.00 to 5.19	3	5.156	4.586	4.730	.735	84.79	920.8	49.8
5.20 to 5.39	3	5.306	5.010	4.833	.804	84.05	965.3	51.9
5.60 to 5.79	2	5.680	5.420	4.490	.812	83.60	1,009.0	53.7
6.00 to 6.19	1	6.00	4.88	4.66	.794	83.67	1,015.7	48.0
JERSEY COWS								
3.20 to 3.39	1	3.280	3.22	3.67	0.789	89.04	631.9	51.0
3.60 to 3.79	5	3.680	3.896	3.942	.729	87.75	713.7	54.6
3.80 to 3.99	8	3.910	3.623	4.860	.683	86.92	755.1	48.0
4.00 to 4.19	5	4.074	3.302	4.866	.660	87.10	752.2	43.9
4.20 to 4.39	11	4.292	3.695	5.126	.697	86.19	806.1	45.8
4.40 to 4.59	18	4.493	3.648	5.058	.691	86.11	819.3	44.5
4.60 to 4.79	16	4.668	3.556	5.059	.681	86.03	830.2	42.8
4.80 to 4.99	20	4.881	3.715	5.058	.693	85.65	859.3	43.2
5.00 to 5.19	19	5.091	3.896	4.919	.694	85.40	883.1	44.1
5.20 to 5.39	26	5.295	3.859	5.026	.698	85.12	904.7	42.7
5.40 to 5.59	16	5.479	3.891	4.986	.693	84.95	921.7	42.2
5.60 to 5.79	16	5.707	4.171	4.963	.743	84.42	959.7	43.5
5.80 to 5.99	12	5.892	4.288	4.929	.732	84.16	981.4	43.7
6.00 to 6.19	8	6.081	4.264	4.764	.726	84.17	990.9	43.0
6.20 to 6.39	5	6.230	4.010	4.982	.713	84.06	999.2	40.1
6.40 to 6.59	4	6.445	4.068	4.788	.727	83.97	1,015.3	40.1
7.60 to 6.79	5	6.666	4.008	5.042	.709	83.58	1,042.1	38.5
7.80 to 6.99	1	6.80	4.44	4.96	.698	83.10	1,074.0	41.3
8.20 to 7.39	1	7.34	4.91	4.06	.650	83.04	1,111.3	44.2
5.40 to 7.59	1	7.59	3.23	5.15	.706	83.32	1,090.7	29.6
8.20 to 8.39	2	8.365	4.925	3.605	.772	82.33	1,195.7	41.2
CROSSBRED COWS								
2.60 to 2.79	1	2.72	3.27	3.91	0.682	89.42	586.5	55.8
2.80 to 2.99	4	2.933	2.915	5.113	.682	88.36	635.7	45.9
3.00 to 3.19	19	3.085	3.103	5.093	.689	88.03	659.6	47.0
3.20 to 3.39	19	3.295	3.203	5.048	.685	87.77	682.6	46.9
3.40 to 3.59	61	3.497	3.466	4.987	.697	87.35	713.8	48.6
3.60 to 3.79	89	3.700	3.514	4.929	.711	87.15	733.7	47.9
3.80 to 3.99	111	3.895	3.560	4.917	.716	86.91	754.2	47.2
4.00 to 4.19	125	4.099	3.622	4.878	.723	86.68	775.4	46.7
4.20 to 4.39	110	4.293	3.790	4.894	.723	86.30	803.2	47.2
4.40 to 4.59	130	4.489	3.864	4.820	.723	86.10	822.6	47.0
4.60 to 4.79	93	4.690	3.918	4.833	.739	85.82	845.6	46.3
4.80 to 4.99	70	4.895	4.046	4.820	.737	85.50	871.0	46.5
5.00 to 5.19	60	5.090	4.149	4.834	.743	85.18	895.7	46.3
5.20 to 5.39	42	5.278	4.275	4.665	.739	85.04	913.1	46.8
5.40 to 5.59	27	5.477	4.480	4.818	.761	84.46	950.0	47.2
5.60 to 5.79	17	5.679	4.422	4.558	.769	84.57	955.7	46.3
5.80 to 5.99	11	5.874	4.735	4.383	.756	84.25	983.1	48.2
6.00 to 6.19	2	6.120	4.835	4.640	.776	83.63	1,022.8	47.3
6.20 to 6.39	4	6.290	4.655	4.658	.769	83.63	1,029.4	45.2
6.40 to 6.59	6	6.472	4.653	4.747	.798	83.33	1,051.3	44.3
7.40 to 7.59	1	7.50	5.54	4.04	.814	82.11	1,167.6	47.4

The equation $E=a+bf$ has been fitted to the average fat-percentage and energy values of each of these tables by the method of least squares, and weighting by the number of records at each fat-percentage class. The equations are given in Table 3, together with the directly determined equations of the investigators referred to at the outset.

TABLE 3.—*Milk-energy formulas for various breeds of cows*

Breed	Formula *	Authority
Not classified	$E=115.33\ (2.51+f)$	Overman and Sanmann.
Red Danish	$E=115\ (2.44+f)$	Möllgaard.
Jersey	$E=101\ (3.59+f)$	Do.
Holstein	$E=128.55\ (1.89+f)$	Kahlenberg and Voris.
Jersey	$E=106.98\ (3.15+f)$	Present paper.
Holstein	$E=128.19\ (1.99+f)$	Do.
Guernsey	$E=116.80\ (2.52+f)$	Do.
Ayrshire	$E=121.00\ (2.20+f)$	Do.
Crossbred	$E=116.11\ (2.61+f)$	Do.

* E=calories per kilogram milk, f=percentage fat content of milk.

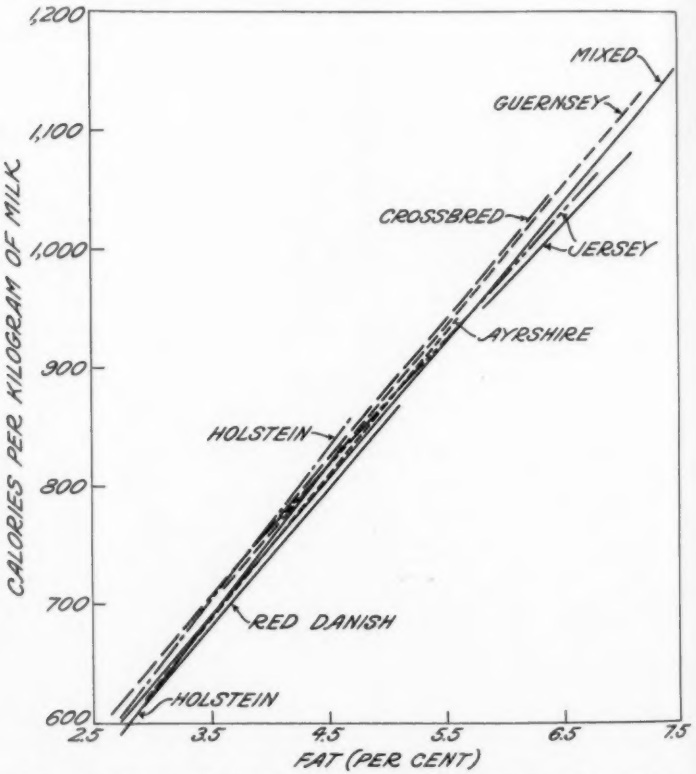

FIGURE 1.—Fitted curves showing relation of milk energy to fat percentage. The broken lines represent data of this paper. Equations in Table 3

A study of this table suggests that there is undoubtedly some difference in the slopes of the breed curves. The Holstein breed has the steepest curve and the Jersey breed the flattest. The slope of the present indirectly determined curve for the Holstein breed agrees very closely with the directly determined data of Kahlenberg and Voris. The similar comparison with Möllgaard's Jersey data shows somewhat less exact agreement, but the results are in accord in indicating a difference between the two breeds.

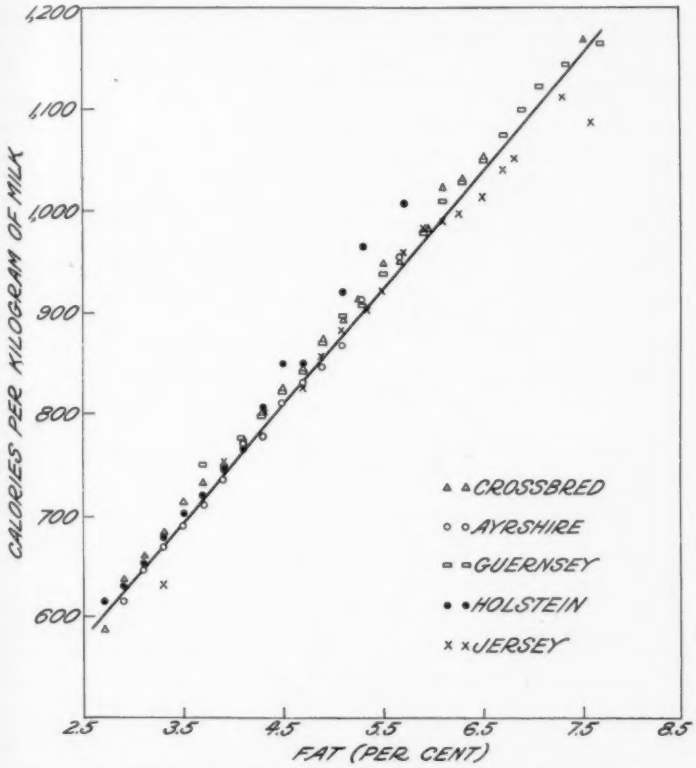

FIGURE 2.—Observations of Table 2 showing relation of milk energy to fat percentage. The line represents Overman and Sanmann's equation

The curves of the equations of Table 3 are plotted in Figure 1, which gives a better general view of the variation between breeds than do the equations. The curves are plotted to cover the range of fat percentage found in the observed data on which they are based. Considered within these limits the curves all lie in a comparatively narrow band with slope practically the same as that of Overman and Sanmann's mixed data. While the difference in breeds may be of real significance, there is no great error involved, within

the usual fat-percentage range of any breed, by using a single equation to describe the fat-energy relation for all breeds.

The computed energy values of Table 2 are plotted against fat percentage in Figure 2. The curve of Overman and Sanmann's equation is also given as a guide. It is apparent that a majority of the observations lie above this line—that is, the equation gives values slightly too low to fit the observed values. This figure brings out a difference between high-testing Holstein and Jersey samples. High-testing Holstein samples tend to show an energy content above that expected, while the high-testing Jersey samples tend to show an energy content below that expected. In general, Figure 2 shows that breed differences are slight for samples testing within the usual fat-percentage range of the breed.

PROTEIN-ENERGY RATIO

Fredericksen (*4*) has pointed out that the ratio of protein to energy is nearly the same regardless of the natural fat-content percentage of the milk. This observation is of prime importance from the point of view of the whole-milk trade and from the point of view of dairy feeding standards. The protein-energy ratio has been computed for each fat-percentage class and is given in the last column of Table 2. The ratios for each breed have been connected with the fat percentage by means of the equations given in the following tabulation. The equations were fitted by the method of least squares weighting by the frequencies.

Breed	Milligrams of protein per calorie	Breed	Milligrams of protein per calorie
Holstein	$46.49+0.46f$	Ayrshire	$56.29-2.32f$
Crossbred	$49.68-.61f$	Jersey	$55.59-2.32f$
Guernsey	$48.89-.83f$	Jersey [6]	$47.93-.87f$

The observed ratios and fitted curves are shown graphically in Figures 3 and 4, and all the fitted curves are brought together in Figure 5. There seem to be marked differences between the breeds in the change of the protein-energy ratio with change in fat percentage. The Holstein breed shows a general tendency for the ratio to increase with increase of fat percentage, while the Ayrshire breed shows a marked decrease in the proportion of protein to energy as the fat percentage increases. It seems safe to say that the Ayrshire and Holstein breeds are significantly different in the protein-energy ratio of their milk. It may be noted that the Holstein observed values (fig. 3) are somewhat irregular, while the Ayrshire values (fig. 4) conform very closely to the fitted curve throughout the range.

According to the fitted curves as shown in Figure 5, the Ayrshire and Jersey breeds resemble each other and stand more or less apart from the other breeds. Reference to Figure 4 suggests, however, that the Jersey curve may be influenced to a considerable degree by the observations below $f=4.10$, and above $f=6.10$. If these observations, representing 33 analyses or 16.5 per cent of the total, are excluded a much flatter curve is obtained, as shown by the broken line in Figures 4 and 5. This limited Jersey curve resembles the Guernsey curve. The data therefore leave one in doubt as to the genuineness of the resemblance of the Jersey curve to the Ayrshire

[6] For limits of $f=4$ to $f=6.19$.

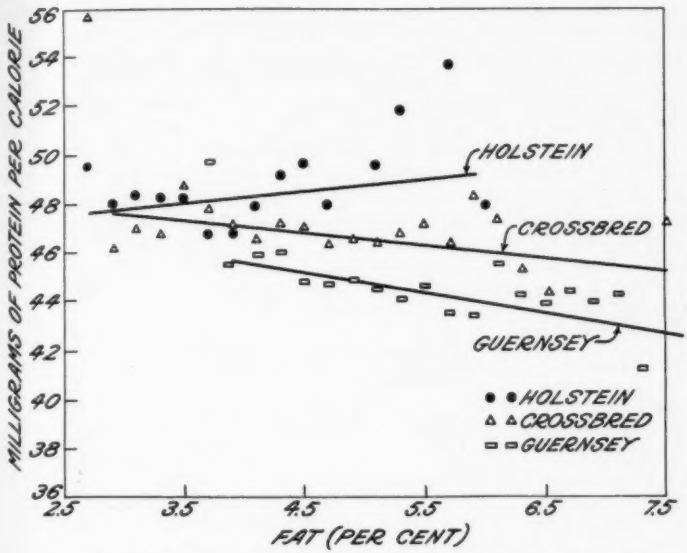

FIGURE 3.—Relation between the protein-energy ratio and the fat percentage of the milk from Guernsey and Holstein breeds and crosses of these two breeds. The lines represent the fitted equations

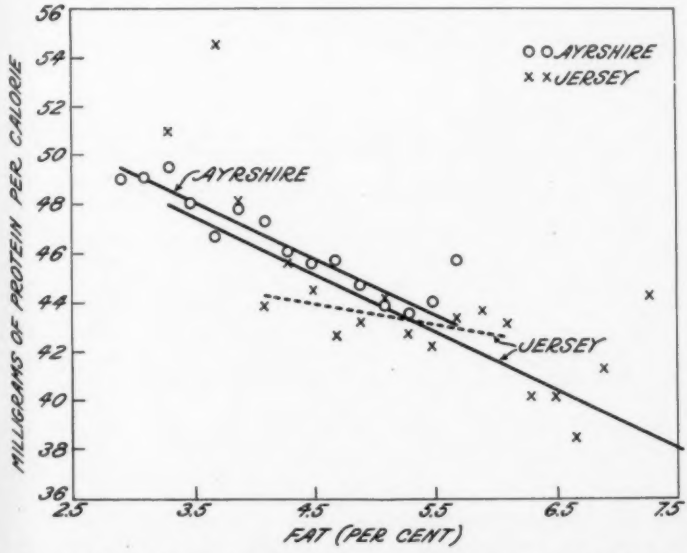

FIGURE 4.—Relation between the protein-energy ratio and the fat percentage of the milk from Ayrshire and Jersey breeds. The solid lines represent the fitted equations; the broken line applies to the Jersey data over the limited fat-percentage range

curve. It would be more natural on the basis of ancestral descent to accept the resemblance to the Guernsey curve.

Generally speaking, the present ratios indicate that the amount of protein in whole-milk samples from cows of all breeds lies between 43 and 49 mg of protein per calorie. If we were to deal with the lactation milk yield of individual cows, or mixed herd milk, this range would undoubtedly be considerably reduced. So far as the whole-milk trade is concerned it is apparent that the reduction of normal whole milk to a calorie basis places it on a food basis not only with respect to total nutrients, but also, substantially, with

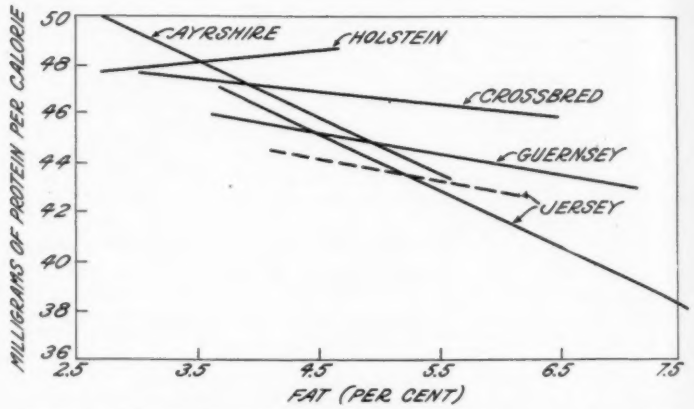

FIGURE 5.—Fitted curves showing the relation between the protein-energy ratio and the fat percentage of milk from different breeds of cattle. The broken line applies to the Jersey data over the limited fat-percentage range. The equations are given in the text

respect to its protein content. So far as feeding standards for milk cows are concerned, it is apparent that on a calorie basis the variation in protein content of the milk is practically negligible.

SIMPLIFIED DAIRY FEEDING STANDARD

The feed requirements for milk production are generally given in table form, listing numerous fat-percentage classes of milk. It has been shown (*5*, *fig. 4*) that the total nutrients required for lactation are closely proportional to the milk energy (Haecker's law). Since the milk protein is also substantially proportional to milk energy, it follows that the main food requirements, total digestible nutrients, and protein can be expressed as very simple functions of the milk energy.

One way of expressing milk energy is in terms of 4 per cent milk by the formula, 4 per cent milk $= 0.4M + 15F$, where M is milk and F is fat, all in the same unit of weight. On the basis of 750 calories per kilogram of 4 per cent milk this formula is practically the same as that of Overman and Sanmann. Figure 1 shows that this formula is an adequate expression for all the breeds from a practical standpoint. One kilogram of 4 per cent milk, natural or by the $0.4M + 15F$ formula, contains 750 calories and 37 g of protein, on the basis of 50 mg

of protein per calorie. Allowing a 100 per cent margin for calories and a 50 per cent margin for protein (in line with current feeding standards), the lactation requirement for 1 kg of 4 per cent milk would be, therefore, 1,500 calories of digestible feed energy and 55 g of digestible protein. This principle is embodied in a plan for the teaching and practice of feeding dairy cows which is widely used in the Scandinavian countries at the present time.

So far as dairy cows alone are concerned, it would appear entirely sufficient to determine for the various feeding stuffs simply the content of energy and protein and the digestibility coefficient for these two items. The feeding value might then be expressed in terms of digestible energy and digestible protein, which as above pointed out, bear a definite ratio to the 4 per cent milk requirements. This would reduce the amount of work necessary in feed analyses and digestion trials.

INHERITANCE ASPECTS

From an inheritance point of view it is interesting to compare the curves of Figures 1 and 5 relating to cows of the Guernsey and Holstein breeds and the crossbred cows resulting from the crossing of these two. With regard to the relation of milk energy to fat percentage the difference between the parental breed curves is not great, and there is consequently little chance for clear-cut results in the cross. The slope of the crossbred curve is the same as that of the Guernsey breed and somewhat less than that of the Holstein breed.

In the case of the protein-energy ratio, however, there is more difference in the parental breeds, and the crossbred cows take more or less distinctly an intermediate position. (Fig. 3.) It should be mentioned that the Guernsey and Holstein samples are not from the actual parents of the crossbred cows, but only from cows of the same breed as the parents.

SUMMARY

Milk-energy formulas for the Ayrshire, Guernsey, Holstein, and Jersey breeds, and Guernsey-Holstein crossbred cows have been derived from analyses available from this laboratory. The analyses (212), including a direct energy determination, were used to determine the energy value of the several milk components. The values were then applied to another, more extensive series of analyses (1,999), and the milk energy was thus indirectly determined. Based on fat, protein, and lactose only, the following values in calories per gram were found: Fat, 9.253 ± 0.065; protein, 5.853 ± 0.127; lactose, 3.693 ± 0.059.

The breed formulas for the estimation of milk energy based on the fat percentage showed small but probably significant differences. As compared with the original formula of Overman and Sanmann the breed formulas, considered within the usual fat-percentage limits, all lay within a narrow band on either side. It is concluded that for practical purposes it is permissible to use a single formula for all breeds, which may be expressed in terms of 4 per cent milk as 4 per cent milk $= 0.4M + 15F$, in which M is the weight of milk and F is the weight of fat, all in the same unit. One kilogram of 4 per cent milk = 750 calories, or perhaps slightly more.

The change in the protein-energy ratio with change in fat percentage showed more marked breed differences than were found for milk energy. Crossbred cows were intermediate as compared with the parent breeds. In a rough way the protein-energy ratio may be considered constant, lying usually within the limits of 43 to 49 mg. of protein per calorie. With respect to protein and energy content the human food value of natural-cows' milk is therefore proportional to the amount of 4 per cent milk by the above formula. For the purpose of a usable feeding standard for milk production, the milk may be converted to a 4 per cent basis by the above energy formula and the digestible energy and protein requirements then expressed as constant multiples of the 4 per cent milk. It is suggested that feeding standards for milk production might be adequately handled by determining only the energy and protein in the feed, together with their digestion coefficients.

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INHERITANCE OF REACTION TO STEM RUST AND BARBING OF AWNS IN BARLEY CROSSES[1]

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INTRODUCTION

Information regarding the mode of inheritance of reaction to certain plant pathogenes aids the plant breeder in developing a logical plan for the improvement of particular crop plants. This paper presents the results of a study of the transmission of reaction to stem rust (*Puccinia graminis tritici* Eriks. and Henn.) in a cross between susceptible and resistant varieties of barley (*Hordeum vulgare* L.). The data were taken in an endeavor to determine the number of factor pairs differentiating resistance and susceptibility and whether these factors are inherited independently of the *Rr* factor pair for rough and smooth awn.

Considerable progress has been made in determining the manner in which resistance to the attacks of stem rust is inherited in the genus Triticum, but the writers have been unable to find references to work of this nature with the genus Hordeum. Since the literature pertaining to Triticum has recently been reviewed by Hayes et al.[3] such a review is not included here.

MATERIALS AND METHODS

The parents used in the crosses were Glabron, Peatland, and Minnesota No. 462. Glabron and Minnesota 462 are smooth-awned sister selections from a cross of Smooth Awn × Manchuria. Peatland is a rough-awned selection from a variety called Switzerland.

Both the F_1 and F_3 generations were grown in 6-foot rows 1 foot apart, the F_1 being spaced 4 inches apart within the row and the F_3, 3 inches. The Glabron and Peatland parents were sown in every tenth and eleventh row, respectively. The F_3 families were from seed of F_2 plants chosen at random.

No artificial epidemic of stem rust was attempted. The field nursery in which the barley was grown was 2 or 3 rods from the spring-wheat rust nursery. In the epidemic induced in spring wheat a large number of physiologic forms of stem rust were used. It is possible that most of the barley infection was a result of the spread of rust from the spring wheat.

In order to identify the physiologic forms present, urediospores were collected from the nearly mature plants of Glabron, Peatland,

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[2] The writers wish to express their sincere appreciation to Dr. H. K. Hayes and Dr. E. C. Stakman for kindly suggestions and criticisms during the course of the experiments and preparation of the manuscript, and to Dr. F. R. Immer for his criticisms of the statistical phases of the paper.

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Minnesota 462, the F_1 generation of Minnesota 462 × Peatland, and susceptible and resistant plants of one of the F_3 families of the Peatland × Glabron cross.

All notes were taken in the field at that stage in the development of the plants when the heads were mature but the culms were still somewhat green. The plants were pulled and classified as to barbing of awns and reaction to stem rust. An attempt was made to separate the rough and smooth-awned plants on the basis of the segregation of the *Rr* factor pair disregarding the *Ss* factor pair (Griffee (*3*)).[4] Likewise only two classes were used in separating the resistant and susceptible plants, since it was impossible to differentiate between the homozygous resistant and the heterozygous resistant individuals.

In analyzing the data, Fisher's (*1*) method of partitioning χ^2 into its components was used, and the values of P are taken from his table of χ^2. Throughout the analyses a value of $P = 0.05$ was taken as the lower level of significance. Values of P equal to or below this level give odds equal to or greater than 19:1 that the deviations noted are not due to errors of random sampling. Such values are considered as statistically significant throughout this study.

EXPERIMENTAL RESULTS

PHYSIOLOGIC FORMS PRESENT

Identification of the physiologic forms of *Puccinia graminis tritici* showed that Nos. 17, 38, and 49 were present. Forms 38 and 49 were isolated from plants of Minnesota 462 and Peatland; Forms 17, 38, and 49 from Glabron, Form 38 from the F_1 generation of Minnesota 462 × Peatland and the resistant F_3 plant of Peatland × Glabron, and Forms 17, 38, and 49 from the susceptible F_3 plant of Peatland × Glabron. It is of interest to note that the susceptible F_3 plant of Peatland × Glabron was infected with the same three physiologic forms as Glabron, whereas from the resistant F_1 generation of Minnesota 462 × Peatland and the resistant F_3 plant of Peatland × Glabron Form 38 only was isolated.

It is probable that the infection was caused by Forms 17, 38, and 49. However, it should be kept in mind that Hayes and others (*5*, *6*, *7*), Harrington (*4*), Stakman (*9*), and Goulden and Neatby (*2*) have shown that mature plants of some varieties of wheat under field conditions possess resistance to many and perhaps all forms of *Puccinia graminis tritici*. On the other hand, it seems probable that physiologic forms of *P. graminis secalis* Eriks. and Henn. were not present, and therefore the present study may not be indicative of the reaction of the crosses and parents to this variety of stem rust.

REACTION TO STEM RUST

An epidemic of stem rust developed on the parents and on the F_1 and F_3 generations. Sixty-three F_3 families from F_2 plants chosen at random were classified for breeding behavior into homozygous resistant, heterozygous, and homozygous susceptible. The accuracy of the classification was measured by the reaction of the Peatland and Glabron parents. Only one plant from the seven rows of the Peatland parent proved susceptible. It is believed that susceptibility in this instance was due to mechanical mixture of the seed, such as may occur in thresh-

[4] Reference is made by number (italic) to Literature Cited, p. 1129.

ing and planting operations. Two of the seven rows of the Glabron parent possessed resistant plants, the frequency in these two rows being 5.6 and 15.4 per cent. These apparently resistant plants probably were genetically susceptible ones which escaped infection by chance.

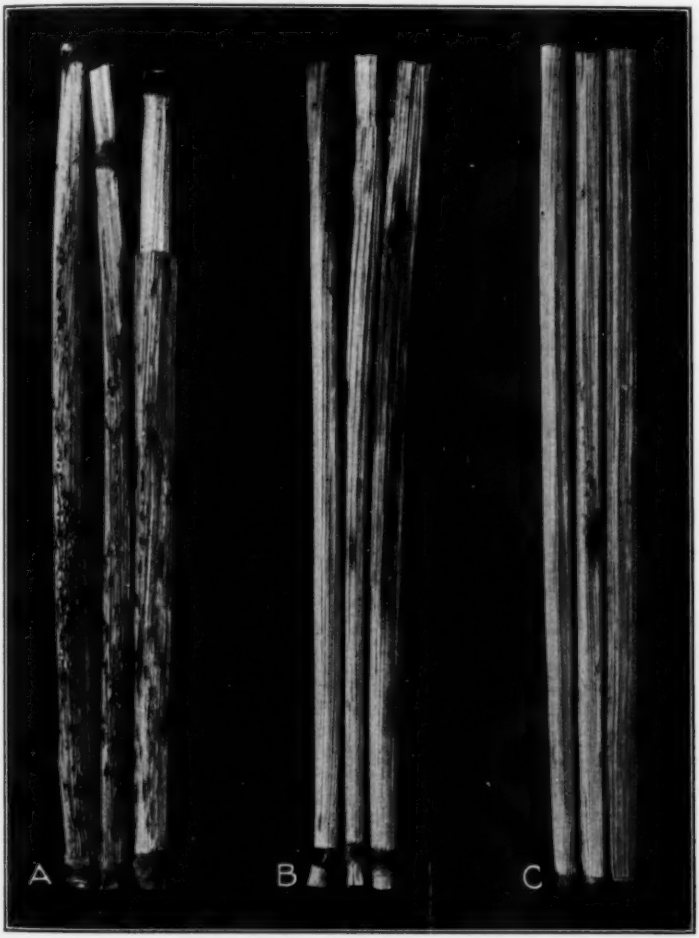

FIGURE 1.—Dominance of resistance to stem rust: A, Susceptible parent, Minnesota 462; B, F_1 generation of a cross between Minnesota 462 and Peatland; C, resistant parent, Peatland

DOMINANCE

It was impossible to distinguish between the heterozygous and the homozygous resistant plants of the second generation, as Figure 1 shows, and therefore the plants were classified by means of the F_3 families. The results obtained in the F_3 generation are illustrated in Figure 2. The culms of Glabron are shown in Figure 2, A, those

from a homozygous susceptible F_3 progeny in 2, B, those from a homozygous resistant F_3 progeny in 2, C, and those of the Peatland parent in 2, D. As in F_2, the plants of the F_3 could be placed only in the two classes, resistant and susceptible, since the heterozygous individuals were indistinguishable from those homozygous for resistance. It may be concluded, therefore, that in this cross resistance is dominant to susceptibility.

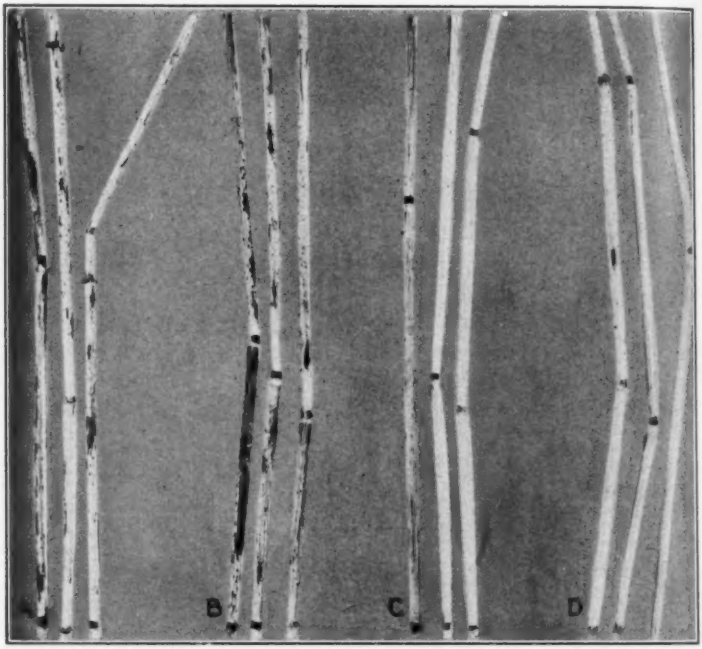

FIGURE 2.—Reaction to stem rust of parents and of a homozygous susceptible and a resistant F_3 plant of a cross between Peatland and Glabron: A, Glabron, susceptible parent; B, susceptible F_3 plant; C, resistant F_3 plant; and D, Peatland, resistant parent

TABLE 1.—*Segregation of the F_2 generation of Peatland × Glabron for reaction to stem rust, the classification being based upon the reaction of F_3 families*

Phenotypic class	Genotype	Number of families obtained	Number of families expected on basis of 1-factor pair difference
Homozygous resistant	*TT*	17	16
Heterozygous	*Tt*	32	31
Homozygous susceptible	*tt*	14	16

Table 1 presents the phenotypes and genotypes of the F_2 generation of the cross Peatland × Glabron as determined from the F_3 families. Statistical analyses are not necessary to show that the numbers

obtained in the three phenotypic classes are very close to those expected on the assumption that Glabron and Peatland are differentiated by a single factor pair. In this connection it should be pointed out that 4 of the 14 families classed as homozygous susceptible had a few resistant plants. The frequency of these resistant plants varied from 5.3 per cent to 16.7 per cent. A reversal of dominance would be necessary to explain resistant plants among those classed as homozygous for susceptibility. However, since resistant plants were found also in two rows of Glabron, it seems probable that those found in the four families grown from susceptible parents are in reality plants that escaped infection and therefore the placing of these four families in the homozygous susceptible class is justifiable. If the alternative were accepted (of putting them in the heterozygous class) a total χ^2 value of 2.8413 with a value of P between 0.20 and 0.10 would be obtained. This value is not statistically significant and therefore the data may be considered as fitting the hypothesis, which assumes that only 1 factor pair is involved in the cross.

It remains to be determined whether segregation within the F_3 families supports the results obtained with the F_2 generation. As Table 1 shows, 32 F_3 families were found to be segregating for reaction to stem rust. These 32 families represented a total of 2,196 plants, of which 1,646 were resistant and 550 were susceptible. Statistical analysis is not necessary to demonstrate that this is very close to the ratio of 1,645 resistant : 549 susceptible, which would be expected if resistance and susceptibility are differentiated by a single factor pair.

However, Kirk and Immer (*8*) have pointed out that a factorial analysis based upon the total number of plants in the different classes disregarding segregation within families is not always reliable, as inherent discrepancies may be covered up. To determine whether such were the case in these studies, the χ^2 test for goodness of fit was applied to the data. The results are shown in Table 2.

TABLE 2.—*χ^2 test for goodness of fit between the reaction of F_3 families to stem rust and the theoretical expected based on a 1-factor pair difference*

Family No.	Resistant	Susceptible	χ^2	P lies between—	Family No.	Resistant	Susceptible	χ^2	P lies between—
1537	50	25	1.0159	0.50 and 0.30	1585	12	5	0.1765	0.70 and 0.50
1540	60	21	.0370	.90 and .80	1587	54	17	.0423	.90 and .80
1542	59	20	.0042	.95 and .90	1589	17	2	2.1228	.20 and .10
1543	67	25	.2319	.70 and .50	1590	13	5	.0741	.80 and .70
1546	54	27	2.5720	.20 and .10	1599	28	6	.9804	.50 and .30
1551	86	24	.5939	.50 and .30	1600	75	21	.5000	.50 and .30
1552	73	26	.0842	.80 and .70	1601	69	32	2.4059	.20 and .10
1554	82	32	.5731	.50 and .30	1604	30	12	.2857	.70 and .50
1557	29	12	.3984	.70 and .50	1606	7	2	.0370	.90 and .80
1564	70	21	.1795	.70 and .50	1610	43	15	.0230	.90 and .80
1573	23	7	.0444	.90 and .80	1611	77	15	3.7101	.10 and .05
1574	32	17	3.5986	.10 and .05	1612	81	23	.4615	.50 and .30
1577	60	27	1.6897	.20 and .10	1615	27	12	.6923	.50 and .30
1578	26	15	2.9350	.10 and .05	1603	34	3	5.6306	.02 and .01
1579	89	21	2.0585	.20 and .10	1538	50	32	8.6016	.01 and .00
1581	76	17	2.2401	.20 and .10	1556	84	11	9.1263	.01 and .00

Total χ^2 value=53.1165.
Deviations of χ^2=2.37×standard error.

An examination of Table 2 reveals the fact that the last 3 families gave values of P lower than 0.05. Due to the large values of χ^2 in these three families, the total χ^2 for all of the F_3 families (53.1165) slightly exceeds the limits of significance. By Fisher's (*1*) method

the deviations noted are 2.37 times the standard error. Since only 3 of the 32 families deviated significantly from the expected, a test to determine whether the frequency distribution of χ^2 values for individual families fits the expected was made. The results of such a test as outlined by Kirk and Immer (*8*) are given in Table 3.

TABLE 3.—*Frequency distribution of χ^2 values for goodness of fit between the reaction of individual F_3 families to stem rust and the theoretical expected based on a 1-factor pair difference*

χ^2	P	Expected	Obtained	$\frac{(O-C)^2}{C}$	χ^2	P	Expected	Obtained	$\frac{(O-C)^2}{C}$
0.0000	1.00				0.4550	0.50			
		0.32					6.40	7	0.0563
.0002	.99				1.0740	.30			
		.32					3.20	0	3.2000
.0006	.98		1	1.5125	1.6420	.20			
		.96					3.20	6	2.4500
.0039	.95				2.7060	.10			
		1.60					1.60		
.0158	.90				3.8410	.05			
		3.20	5	1.0125			.96		
.0642	.80				5.4120	.02		3	.0001
		3.20	2	.4500			.32		
.1480	.70				6.6350	.01			
		6.40	5	.3063			.32		

χ^2=8.9877.
P lies between 0.30 and 0.20.

Because of the small numbers, the upper four expected values and the lower four were each grouped into one class. The χ^2 value obtained was 8.9877, which has an accompanying P value between 0.30 and 0.20. Probably the three exceptional families may be accounted for by chance alone, and the segregation of F_3 families for reaction to stem rust is that expected if the Glabron and Peatland parents differ by a single factor pair. Since the results from both the F_2 and the F_3 generations indicate that the parent varieties differ in their reaction to stem rust by a single factor pair, the genotype of Peatland is designated *TT* and that of Glabron *tt*. The letter *T* is taken from *tritici*, because the physiologic forms to which Peatland is resistant are classified as *Puccinia graminis tritici*.

INDEPENDENCE OF INHERITANCE OF REACTION TO STEM RUST AND BARBING OF AWNS

The results of studies to determine the linkage relationships between reaction to stem rust and barbing of awns are presented in two parts: (1) The evidence furnished by the genotypes of the F_2 plants as determined by the breeding behavior of the F_3 families, and (2) an analysis of the F_3 families that segregated for both reaction to stem rust and barbing of awns.

EVIDENCE FURNISHED BY THE F_2 GENERATION GENOTYPES

To determine whether the F_2 generation segregated in the ratio of 9:3:3:1, as would be expected if the *RR* and *TT* factor pairs are not linked, the χ^2 test for goodness of fit was applied to the data. A χ^2 value of 4.0758 was obtained, giving a value for P between 0.30 and 0.20. A more detailed study was made by applying the χ^2 test to a classification of the F_2 generation based on the nine different genotypes expected. The results, summarized in Table 4, show 31 rough-awned and resistant, 10 rough-awned and susceptible, 18 smooth-awned and resistant, 4 smooth-awned and susceptible, which is very close to the ratio 32:9:17:5, which would be expected on the basis of independent inheritance. A P value lying between 0.10 and 0.05 was obtained. Considering that there were only 63 F_3 families on which to classify the F_2 generation, the fit between the expected and the obtained is good.

So far as the F_2 generation is concerned, therefore, the evidence sustains the hypothesis that reaction to stem rust and barbing of awns are independently inherited.

TABLE 4.—χ^2 *for goodness of fit to test whether the number of individuals obtained in the nine different* F_2 *generation genotypic classes vary significantly from the expected*

Genotype	Phenotype	Obtained	Expected	$\frac{(O-C)^2}{C}$
RRTT	Rough awn and resistant	2	3.9375	0.9534
RRTt	do	13	7.8750	3.3353
RrTT	do	7	7.8750	.0972
RrTt	do	9	15.7500	2.8929
RRtt	Rough awn and susceptible	1	3.9375	2.1915
Rrtt	do	9	7.8750	.1607
rrTT	Smooth awn and resistant	8	3.9375	4.1915
rrTt	do	10	7.8750	.5734
rrtt	Smooth awn and susceptible	4	3.9375	.0010

$\chi^2 = 14.3969$.
P lies between 0.10 and 0.05.

Evidence Furnished by Analysis of F_3 Families

An analysis of the F_3 families segregating for both characters permits a study of considerably larger numbers of plants (767) than the F_2; hence the results are more reliable.

In studying the F_3 data it is well to keep in mind that should there be linkage between *RR* and *TT* due to crossing over in the F_1, some of the F_3 families would represent the coupling phase and others the repulsion phase. Since the original cross was in the coupling phase the closer the linkage the greater should be the preponderance of families in this phase. As both the coupling and repulsion phases may be present it seemed desirable to apply to each F_3 family a χ^2 test for goodness of fit to a 9:3:3:1 ratio. When this was done it was found that 4 of the 10 families segregating for both factor pairs deviated significantly from the theoretically expected ratio of 9:3:3:1. (Table 5.)

TABLE 5.—χ^2 *for goodness of fit to a 9:3:3:1 ratio of those* F_3 *families segregating for barbing of awns and reaction to stem rust, partitioned into its components* [1]

Family No.	Ratio obtained	χ^2 for goodness of fit to a 9:3:3:1 ratio	χ^2 for goodness of fit to a 3:1 ratio		Interaction between type of awn and reaction to stem rust
			Rough *v.* smooth	Resistant *v.* susceptible	
1537	37:17:22:8	6.2439	5.1429	1.0159	0.0851
1579	64:18:25:3	3.5192	.0121	2.0485	1.4586
1585	9:3:3:2	.8824	.1765	.1765	.5294
1581	64:14:12:3	6.3453	3.9032	2.2401	.2020
1601	50:21:19:11	4.5226	1.1914	2.4059	.9253
1610	35:14:8:1	3.7318	2.7816	.0230	.9272
1543	62:21:5:4	12.0773	11.3623	.2319	.4831
1556	79:8:5:3	28.3029	13.9263	9.1263	5.2503
1573	11:4:12:3	10.4148	10.0000	.0444	.3704
1577	54:25:6:2	13.8429	11.5900	1.6897	.5632
Total		89.8831	60.0863	19.0022	10.7946

[1] Levels of significance (Fisher (*1*)) $n=1$ is 3.841, $n=10$ is 18.307, $n=30$ is 43.773.

These results made it expedient to analyze the F_3 families further. This was accomplished by partitioning χ^2 into its components. The method employed was similar to that outlined by Fisher (*1*) for backcross data. Since the ratio of rough to smooth type of awn is entirely independent of the ratio of resistant to susceptible plants, that part of the deviations not due to segregation for barbing of awn and for reaction to stem rust must be due to linkage between *Rr* and *Tt*.

Tests for goodness of fit to a 3:1 ratio for segregation of barbing of awns of these 10 F_3 families were made by means of χ^2. Likewise tests for goodness of fit to a 3:1 ratio for segregation of F_3 families into resistant and susceptible classes were made. The interaction between type of awns and stem rust was obtained by subtracting the χ^2 due to segregation for barbing of awns plus the χ^2 due to segregation for reaction to stem rust from the total χ^2 of each F_3 families for goodness of fit to a 9:3:3:1 ratio. The values obtained for the interaction between type of awn and reaction to stem rust should approximate those obtained from a χ^2 test for independent inheritance.

Table 5 shows that with the exception of family 1556 all of the significant χ^2 values are found in the column headed "Rough *v.* smooth." The segregation is definitely not that to be expected on the basis of a 1-factor pair difference between rough and smooth-awned types. Since the classification for barbing of awns was made without the aid of the binoculars it is believed that the deviations noted in five of the families are due to the *Ss* factor pair (Griffee (*3*)), which also affects barbing of awns. A total value of 10.7946 was obtained for the interaction between type of awn and reaction to stem rust. This value is not significant, and therefore, except possibly in the case of family 1556, it does not seem that linkage is responsible for the χ^2 for goodness of fit of F_3 progenies to a 9:3:3:1 ratio being significant. The method employed of determining whether two characters are independently inherited is superior to the χ^2 test for independence since considerably more information may be derived from the same amount of data.

The data for the F_2 and F_3 generations support the hypothesis that in the cross between Peatland and Glabron reaction to stem rust and barbing of awns are inherited independently of each other.

SUMMARY AND CONCLUSION

Inheritance of resistance to stem rust of wheat and barbing of awns was studied in crosses of barley.

Identification studies showed that the physiologic forms of *Puccinia graminis tritici* causing the stem rust were Nos. 17, 38, and 49.

Resistance was found to be dominant to susceptibility.

The reaction to stem rust of Peatland, a resistant parent, and Glabron, a susceptible parent, was differentiated by a single factor pair. This factor pair was designated *Tt*.

An analysis of F_2 genotypes and of 10 F_3 families segregating for barbing of awns (*Rr*) and reaction to stem rust (*Tt*) showed that these two factor pairs are not linked.

Since reaction to stem rust is differentiated by a single factor pair and is not genetically linked with barbing of awns, it should be relatively easy to produce a smooth-awned, high yielding variety of barley resistant to physiologic forms of stem rust Nos. 17, 38, and 49

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LABORATORY TESTS WITH VARIOUS FUMIGANTS ON CODLING-MOTH LARVAE[1]

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INTRODUCTION

The rapid increase in codling-moth infestation from year to year in the aging apple orchards of New Jersey and other States offers a difficult problem to the orchardist. The standard spray of lead arsenate often fails to give efficient control of this pest because of the overwhelming numbers of larvae appearing during the hatching periods of the first and second broods. More efficient control has been obtained by the addition of white-oil emulsion or fish oil to the lead arsenate, the oil acting as a sticker for the lead arsenate as well as an ovicide for the codling-moth eggs. The number of summer oil sprays, however, which can be used without injury to apple trees is limited to about 2 or at most 3, during 1 season. Even with this limited number of oil sprays, injury to foliage and fruit has been observed (*1, 5*)[2] in New Jersey. While searching for other means of controlling this pest, Dr. T. J. Headlee considered the possibility of fumigating apple trees in the dormant stage with liquid hydrocyanic acid or other suitable fumigants. Following his suggestion, the writer carried out fumigation tests in the laboratory with some of the outstanding fumigants in order to determine their toxicity to codling-moth larvae. The results are presented in this paper.

DESCRIPTION OF FUMIGANTS USED

Hydrocyanic acid (HCN) in liquid form is colorless, miscible with water in all proportions, boils at 26.5° C., and has a specific gravity of 0.702 at 16°. Its vapors are very volatile, inflammable, and extremely poisonous to man. Great precaution should be observed in handling it. Since 1916 it has been extensively used (*2, 3, 6, 7, 8*) in fumigating citrus trees against scale.

Ethylene chlorhydrin (C_2H_5OCl) is a colorless liquid, soluble in water, possesses an ethereal odor, boils at 128° C. and has a specific gravity of 1.213 at 20°.

Ethelyne dichloride ($C_2H_4Cl_2$) is a colorless liquid with chloroformlike odor, insoluble in water, boils at 83.5° C., and has a specific gravity of 1.254 at 20°. The two aforementioned compounds were tested by Roark and Cotton (*4*).

Ethylene oxide (C_2H_4O) is a colorless liquid, boiling at 14° C., gaseous at room temperature, soluble in water, and has a specific gravity of 0.887 at 7°. The concentrated vapors are inflammable but practically noninjurious to man. The fumigating value of this compound was first reported by Roark and Cotton (*4*).

Diethylene oxide ($(C_2H_4)_2O$ is a colorless, volatile liquid, boils at 101° C., has a specific gravity of 1.037 at 20°, and is soluble in water in all proportions.

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[2] Reference is made by number (italic) to Literature Cited, p. 1136.

Ethyl acetate ($C_4H_8O_2$) is a colorless liquid of strong ethereal odor, soluble in water, boils at 77° C., and has a specific gravity of 0.9 at 20°. Vapors of ethyl acetate were found by Roark and Cotton (*4*) to be toxic against grain weevil.

Carbon bisulphide (CS_2) is a colorless liquid of 1.29 specific gravity. Its vapor is 2.5 times as heavy as air and highly explosive when mixed with 3 times its volume of oxygen. It boils at 46.1° C., and is only slightly soluble in water. It possesses high toxicity to insects, both in adult and larval stages.

EXPERIMENTAL METHODS

All the chemicals tested were applied in liquid form and were allowed to vaporize in the fumigating chamber. The insects, in numbers of 20 or more, were placed in wire cages and transferred to the fumigating chambers. The chemicals were then added by the use of a pipette, and the chamber was tightly closed. After 1 hour the insects were removed from the chamber and kept for 24 hours in the laboratory before the final observations were made.

A period of 24 hours for final observations was found necessary in order to avoid erroneous results. Preliminary tests showed that some larvae, appearing dead immediately after the test, partially recovered after several hours exposure to the air. This behavior was especially noticeable when hydrocyanic acid was used as the fumigant. On the other hand, some larvae, which were alive when removed from the fumigating chamber, died a few hours later. The mortality was especially pronounced when ethylene chlorhydrin was used.

For all the chemicals tested, except the liquid hydrocyanic acid, large glass bottles of approximately 20-l capacity (19,750 cc) were used as fumigating chambers. The tests were carried out in the following manner: Wire cages containing the insects were placed in the glass container. The chemicals were applied from a pipette on strips of cheesecloth suspended from rubber stoppers. The cheesecloth absorbed and spread the chemical over a large surface, thus aiding in rapid vaporization. The bottle was then tightly stoppered and allowed to stand for 1 hour, with frequent shaking to produce equal distribution of the vapors. After 1 hour the cages were removed and exposed to laboratory temperature for 24 hours. The insects were then removed and examined. The bottles were thoroughly aerated between tests.

A special fumigating chamber was prepared for the hydrocyanic acid on account of the danger in handling it. This consisted of a round container, having a capacity of 5 cubic feet and made of block tin. An opening about 3 inches in diameter, made airtight by the aid of a top screw and lid, was provided on the top for introducing the insects and fumigant. Two small openings fitted with stopcocks, one on the top and one on the side near the bottom, were used for aerating the chamber after each test. In the tests with hydrocyanic acid gas the cages containing the insects were first transferred to the fumigating chamber. A small sheet of asbestos, heated to about 100° C. was then placed in the chamber for the purpose of causing rapid evolution of the gas, and the desired amount of liquid hydrocyanic acid was transferred from a pipette (especially prepared for this purpose) to the hot sheet. The upper opening was closed quickly and made airtight. In order to produce an equal distribution of the gas the container was shaken several times during the period of testing. At the end of this period the chamber was thoroughly aerated by

passing air under 30 pounds pressure through it for 5 to 10 minutes. The insects were then removed, transferred to new cages, and kept in the laboratory for 24 hours when the final observations were made.

By this method seven of the outstanding fumigants were tested on codling-moth larvae, both "naked" and in hibernacula. The larvae were obtained from either burlap or corrugated paper bands removed from apple trees. For the "naked" tests the larvae were completely freed from their hibernacula before being transferred to the cages. The number of larvae used in each test varied from 50 to 200.

The minimum dosages of the various fumigants, calculated in cubic centimeters of liquid per 100 cubic feet of space, required to kill 75 per cent or more of the larvae at laboratory temperatures were first determined. A comparison of the data in Table 1 shows that ethylene chlorhydrin and hydrocyanic acid proved most toxic, with minimum dosages of 20 and 30 cc, respectively. Carbon bisulphide and ethyl acetate ranked very low in toxicity, with minimum dosages of 286 and 572 cc, respectively. Diethylene oxide, ethylene oxide, and ethylene dichloride were midway between the two extremes, with minimum dosages of 115, 143, and 215 cc, respectively.

TABLE 1.—*Minimum dosages of various fumigants required to kill 75 per cent or more codling-moth larvae at laboratory temperatures*

Fumigant used	Chemical formula	Temperature (°F.)	Relative humidity	Quantity used per 100 cubic feet	Total larvae tested	Dead after 24 hours	
						Naked	In hibernacula
			Per cent	*cc*	*Number*	*Per cent*	*Per cent*
Ethylene chlorhydrin	C_2H_5OCl	76	51	20	100	75	----
		76	51	36	100	----	78
Hydrocyanic acid	HCN	68	61	30	200	90	----
		68	61	60	200	----	78
Ethylene oxide	C_2H_4O	70	40	143	50	90	----
		70	40	572	50	----	100
Ethylene dichloride	$C_2H_4Cl_2$	75	30	215	50	80	----
		75	30	286	50	----	76
Diethylene oxide	$(C_2H_4)_2O$	74	50	115	50	92	----
Carbon bisulphide	CS_2	74	30	286	100	80	----
		74	30	572	50	----	91
Ethyl acetate	$C_4H_8O_2$	76	32	572	50	80	----
		76	32	1,174	50	----	78
None (check)	----	----	----	----	100	2	----
		----	----	----	100	----	None

These results indicate that ethylene chlorhydrin and hydrocyanic acid gas are very toxic to codling-moth larvae. It was, therefore, deemed advisable to test these two compounds further at various temperatures. Three sets of temperatures were selected as follows: 40° F., or lower; 50° to 60°; and 60°, or higher. For the two lower sets of temperatures advantage was taken of the exceptionally mild fall of 1931, and the tests were carried out in an open insectary. During the latter part of November and the early part of December the morning temperatures were just above freezing and gradually rose during the day to about 60° or higher. Tests under the higher set of temperatures were carried out in the laboratory.

TABLE 2.—*Tests with ethylene chlorhydrin on naked codling-moth larvae and larvae in the hibernacula fumigated for 1 hour at different temperatures*

NAKED LARVAE

Temperature (°F.)	Relative humidity	Fumigant used per 100 cubic feet	Total larvae tested	Larvae dead after 24 hours
	Per cent	*cc*	*Number*	*Per cent*
68–72	32–51	10	60	62
		20	100	83
		30	50	100
		70	50	100
		140	50	100
50–56	50	10	60	60
	50	20	50	72
	50	40	50	100
	52	70	60	100
	52	140	50	100
37–40	52	20	50	6
	56	40	50	28
	56	70	50	100
	50	140	50	100

LARVAE IN HIBERNACULA

54–62	29–46	20	50	40
		30	50	53
		40	50	100
		70	60	100
		140	60	100
37–40	30–56	20	60	0
		40	60	0
		70	60	94
		140	60	100

TOXICITY OF ETHYLENE CHLORHYDRIN AT DIFFERENT TEMPERATURES

The results on naked larvae, presented in table 2, indicate that the toxicity of ethylene chlorhydrin is not changed at temperatures varying from 50° to 72° F. At this wide range of temperatures 30 cc per 100 cubic feet was the minimum dosage required to kill 100 per cent larvae. At temperatures of 40° or lower, the toxicity of ethylene chlorhydrin was decidedly decreased, a dosage of 70 cc per 100 cubic feet being required to produce 100 per cent kill. When tested on larvae in hibernacula (Table 2) a dosage of 40 cc per 100 cubic feet of space was sufficient to produce 100 per cent kill, at temperatures of 54° to 62°, whereas more than three times this quantity (140 cc) was required to kill 100 per cent of the larvae at temperatures of 37° to 40°.

TOXICITY OF HYDROCYANIC ACID GAS AT DIFFERENT TEMPERATURES

Results similar to those obtained with ethylene chlorhydrin were also obtained with hydrocyanic acid gas (Table 3). Tests on naked larvae showed no differences in toxicity between temperatures of 54° to 72° F., 40 cc per 100 cubic feet being the minimum dosage required to kill from 90 to 100 per cent of the larvae. At a temperature of 40°, or lower, however, the toxicity was greatly reduced and 80 cc of the gas gave only about 30 per cent kill. When tested on larvae in hibernacula, no appreciable differences in toxicity were observed at temperatures ranging from 54° to 64°, a dosage of 30 cc per

100 cubic feet giving 92 to 100 per cent kill of larvae. On the other, hand, at 40° or lower a dosage of 80 cc gave 30 per cent kill, whereas a dosage of 160 cc killed only 44 per cent of the larvae.

TABLE 3.—*Tests with hydrocyanic acid gas on naked codling moth larvae, and larvae in the hibernacula, fumigated for 1 hour at different temperatures*

NAKED LARVAE

Volume of fumigant used per 100 cubic feet	Total of larvae tested	Larvae dead after 24 hours	Temperature (°F.)	Relative humidity
Cc	*Number*	*Per cent*		*Per cent*
20	50	57	64–72	48–60
40	100	96		
80	100	100		
20	100	64	54–56	90
40	100	98		90
80	100	100		46
20	100	20	34–40	79
40	100	36		82
80	100	30		63
40	100	63	60–62	61
40	100	42	48–52	58
80	200	96	58–60	80
80	200	100	64	54
80	100	92	54	56
40	100	22	36–40	75
80	100	30		72
160	100	44		68

The results presented in the tables clearly indicate that at a temperature of 50° F., or higher, both hydrocyanic acid and ethylene chlorhydrin are more toxic to codling-moth larvae than at a temperature of 40°, or lower. These differences may be explained in two ways: (1) Both fumigants volatilize very slowly at low temperatures, and not enough gas accumulates within 1 hour to produce a lethal concentration. Even where the evolution of the hydrocyanic acid gas was hastened by the aid of a hot asbestos sheet, the low temperature in the fumigating chamber would tend to condense the gas back into its liquid form and thus decrease its rates of diffusion. (2) At lower temperatures the insects are less active than at higher temperatures and may not be so readily susceptible to the poisonous action of the fumigants.

SUMMARY

The following fumigants were tested on codling-moth larvae at different temperatures under laboratory conditions: Liquid hydrocyanic acid, ethylene chlorhydrin, carbon bisulphide, ethyl acetate, ethylene oxide, diethylene oxide, and ethylene dichloride. The minimum dosages, calculated in cubic centimeter of liquid per 100 cubic feet of space, required to kill 75 per cent or more of larvae were determined.

The results from tests on naked larvae show that ethylene chlorhydrin and hydrocyanic acid proved most toxic, the minimum dosages being 20 cc and 30 cc, respectively. Carbon bisulphide and ethyl acetate ranked very low in toxicity, the minimum dosages being 286 cc and 572 cc, respectively. Diethylene oxide, ethylene oxide, and ethylene dichloride were midway between the two extremes, with

minimum dosages of 115, 143, and 215 cc, respectively. The tests on larvae in hibernacula show, in general, the same order of toxicity for the various fumigants as observed on naked larvae, with the exception that the minimum dosages were considerably larger. Low temperatures greatly decreased the percentage of kill obtained with both hydrocyanic acid and ethylene chlorhydrin.

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APPARATUS FOR MAKING AUTOGRAPHIC RECORDS OF CATALASE ACTIVITY OF PLANT TISSUES, AND THE PROCEDURE INVOLVED[1]

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INTRODUCTION

Variations in activity of the enzyme catalase, which is universally present in living tissue, may be a useful indicator of certain metabolic conditions of plants and animals. In the case of apple-tree tissues, for example, catalase activity of the leaves or of the bark affords a convenient and highly sensitive index to some changes in the internal condition of the plant, which in turn may be related to such manifestations as vigor of shoot growth, flower-bud formation, condition of maturity, resistance to winter injury, and the like. Similar relations between catalase and certain growth phenomena are found in other plants and tissues, including seeds and fruits (*1*, *2*, *3*, *6*, *7*, *8*).[2]

While the determination of catalase activity is relatively simple, there is no widely adopted standard procedure. The results obtained by different workers depend to a large extent upon the methods used. The apparatus described in this article is an elaboration of the device illustrated in Cornell Memoir 74 (*4*). Its chief features are that it enables the simultaneous determination of catalase activity in 12 different samples of tissue with an autographic record of the results in each case. In using this equipment there is a great saving of time wherever numerous determinations are to be made at frequent intervals. The graphic records afford an accurate and convenient means of comparing the catalase activity of different tissues or of the same tissue at different seasons.

DESCRIPTION OF APPARATUS

Figure 1 shows the complete apparatus with the 12 reaction chambers in use. The 2-arm reaction tubes (*k*) and the gas-collecting bulbs (*l*) are immersed in a copper water bath (*c*), which normally occupies the position outlined by the dashes. The bath is kept at a constant temperature by means of an electric heating unit controlled by a thermoregulator. The motion of the shaker (*a*) agitates the water, thereby insuring a uniform temperature throughout.

The shaking device, which also supports the reaction tubes, is made of brass and is operated by an electric motor. The rate of vibration of the shaker and the distance to and fro may be governed by varying the diameter of the pulley and the length of the levers. (Fig. 1, *a'*.) When the tubes are held on the support by the spring clamps they must be tilted forward. Such a position provides a

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[2] Reference is made by number (italic) to Literature Cited, p. 1143.

greater exposure of surface of the reacting solutions when the shaker is in operation. It is thus possible to provide thorough mixing of the liquids in the reaction tubes without the accumulation of all material alternately in one arm or the other. This insures the free release of oxygen at a regular rate.

The gas-collecting bulbs are held firmly in place by means of a special brass support. (Figs. 1, *b* and 2, *b*.) This support may be moved along rods which are fastened to the side of the water bath. (Fig. 1, *b'*.) Each gas bulb is connected to a reaction chamber by means of heavy rubber tubing. The rubber tube is just long enough to permit free motion of the shaker.

The details of connecting reaction tubes, gas bulbs, and burettes are clearly shown in Figure 1 and are indicated diagrammatically in Figure 2. The water, which is forced out of the gas bulbs (fig. 2, *a*, *b*)

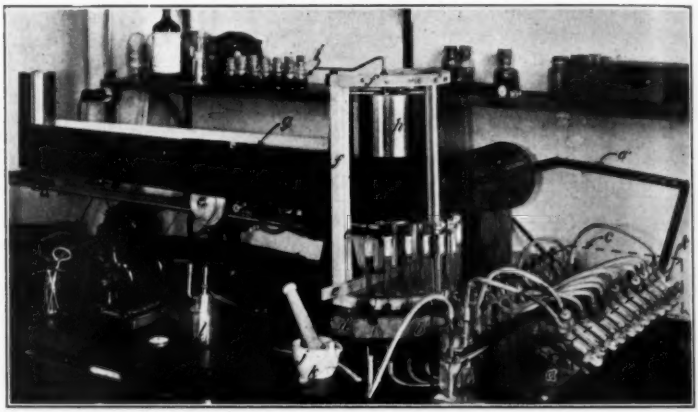

FIGURE 1.—General view of apparatus used for autographic records of catalase activity: *a*, *a'*, Brass shaking device and support for reaction chambers; *b*, *b'*, brass support for gas-collecting bulbs; *c*, position of constant temperature bath; *d*, base of wooden support upon which are mounted the burettes with floating indicator pens; *e*, movable support with guide wires by means of which pens may be held away from smoked paper; *f*, support for plumb lines, with spiral weight, which hold pens against smoked paper; *g*, smoked paper for autographic record of determination; *h*, revolving drum of kymograph; *i*, small bottles containing macerated tissue with prepared chalk and distilled water added; *j*, small mortar and large pestle used in triturated tissue; *k*, 2-arm tubes; *l*, gas-collecting bulbs used as reaction chambers

as the volume of oxygen increases as a result of the catalase reaction, is transferred to the glass tubes or burettes (fig. 2, A, *e*) containing the floating indicator pen. (Fig. 2, A, *f*.) These burettes are about 12 mm in internal diameter and are selected for uniformity of bore. They are firmly mounted with rubber gaskets in holes drilled at right angles in the thick wooden support. (Figs. 2, A, *d* and 2, C, *a*.) This support may be adjusted in any direction so as to permit proper leveling and alignment. The indicator pen in each tube is floated by a cork (fig. 2, A, *g*), saturated with paraffin, and is held in an upright position by the metal cap. (Fig. 2, A, *h*.) The indicator is made of stiff wire and consists of an upright member and a horizontal arm (fig. 2, A, *i*) whose length and position may be adjusted by moving the wires through the holes in the diamond-shaped metal union.

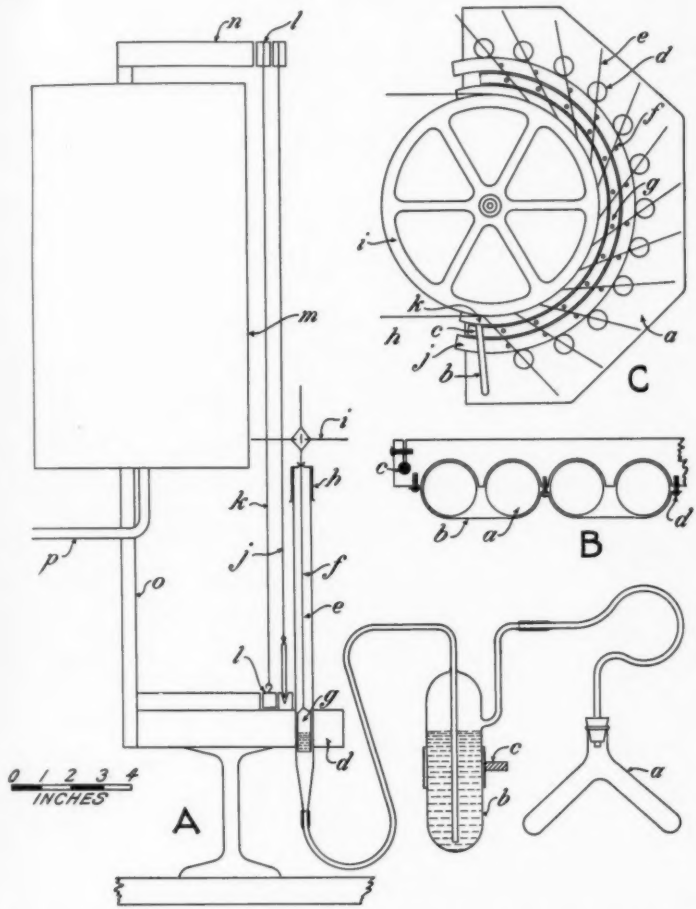

FIGURE 2.—A, Diagram of apparatus for autographic records of catalase determination, showing connections and longitudinal section of recording device: *a*, Reaction tubes, capacity of each arm about 10 cc and total volume 40 cc; *b*, gas-collecting bulbs containing water, connected with *a* by heavy rubber tubing; *c*, cross section of brass support (top view shown in B); *d*, support for burettes and recording device; *e*, glass burette connected with *b* by heavy rubber tube; *f*, floating pen; *g*, cork float impregnated with paraffin; *h*, metal cap to guide indicator; *i*, horizontal arm which may be adjusted in any direction by moving through the small diamond-shaped metal connection plate; *j*, plumb line of fine wire with lead weight to hold pen against drum; *k*, taut wire stretched on support to hold pens away from drum; *l*, movable support for holding taut guide wires; *m*, drum of kymograph; *n*, upper part of support; *p*, support for drum. B, Brass support for holding gas-collecting bulbs: *a*, Cross section of bulb; *b*, metal tape; *c*, rod along which support may be moved up or down; *d*, screws for adjusting pressure of tape. C, Arrangement of indicating units as viewed from above: *a*, Base of heavy wooden support; *b*, lever for operating support; *c*, support which moves in track between *j* and *k*; *d*, cross section of burettes; *e*, horizontal arm of floating pen which moves vertically with change in level of liquid in burettes; *f*, plumb lines serving as guide wire to hold pen against drum; *g*, taut wires attached to movable support which hold pens away from drum when lever is pulled to left of position shown in diagram; *h*, smoked paper; *i*, revolving drum

When in operation, the horizontal arm of the indicator is held against the paper on the recording drum (fig. 2, A, *m*) by means of No. 36 constantan wire (fig. 2, A, *j*) which is suspended from the support (figs. 1, *f*, and 2, A, *n*). This wire is held taut by the lead weight attached to the lower free end. Care must be taken in suspending these plumb lines so that the pressure exerted against the arms is not great enough to interfere with the vertical movement of the indicator as it is forced up by the float. In the plan shown in figure 2, C, the small dots represent the points at which the plumb lines come in contact with the pens.

When not in use, the arms of the floating pens are held away from the drum by means of the guide wires (figs. 2, A, *k*, and 2, C, *g*) attached at the upper and lower ends of that part of the support which moves in a semi-circular groove and which is operated by a lever (figs. 2, A, *l*, and 2, C, *b* and *c*).

The smoked paper upon which the catalase activity of the sample is graphically recorded is fixed to the revolving drum of the kymograph. (Fig. 1, *g* and *h*.) The horizontal lines on the paper represent cubic centimeters of oxygen developed during the reaction. In calibrating the burettes to determine the exact spacing of these lines, successive cubic centimeters of air are added to the reaction tube through a 2-hole stopper substituted for that purpose. It is of course easy to determine whether the various units give comparable results. When equal amounts of the same solution are used in each of the 12 tubes, the curves obtained are identical in a proper set-up under standard conditions.

The kymograph is mounted on a grooved steel track, fastened to a heavy table. It may be pulled away from the support containing the indicating devices whenever the smoked paper is to be changed. The drum is operated by an electric motor and may be run at a wide range of speeds. Active as well as inactive samples may therefore be accommodated without taking up too much space of the chart. The full length of the chart should accommodate 8 sets each of 12 determinations, or 96 separate graphical records. The actual running time is recorded by means of a stop watch so that the graph may be translated in terms of volume and time. The running time, nature of sample, and any other pertinent data may be written on the chart, and the entire record may be rendered permanent by dipping the chart in varnish.

STANDARD PROCEDURE

In using the equipment described above, the following procedure has been found convenient for studies involving fruit-tree tissues and is the standard method used in work previously reported by the author (*4*, *5*). The amount of tissue involved need not exceed 0.1 g fresh weight. This is furnished, for example, by 5 to 10 buds, by 20 disks of leaf tissue each 7 mm in diameter, or by 3 to 5 disks of bark each 5 mm in diameter. Larger amounts of tissue may, of course be used to obtain composite samples or for other purposes. A sharp cork borer is well adapted for sampling such tissues as leaves or bark. Unavoidable injury in sampling, such as occurs in cutting the bits of tissue, should be kept at a minimum and should be uniform.

The fresh tissue is held in 100-cc tightly stoppered bottles and is weighed as soon as possible after collection. After the sample is weighed, it is placed in a small mortar and moistened with about 1 to 2 cc of distilled water. Enough precipitated chalk is added to form a creamy mixture with which the tissue is thoroughly coated before trituration begins. It has been demonstrated that catalase activity may be greatly inhibited in some preparations unless neutralization occurs as soon as the cells are ruptured. In this connection it should be pointed out that the precipitated chalk is in a much finer state of division than are the ordinary samples of chemically pure powdered calcium carbonate, and probably for this reason the neutralization seems to be more effective in the former case. After thorough trituration with a large pestle (a bit of quartz being added when woody tissues are involved), enough distilled water is added to give a final dilution of 1 g of fresh material in 100 cc of water for active tissue, such as leaves or buds, and 1 to 50 for less active samples, such as bark or wood. The diluted preparations are returned to the small bottles and kept in a cool place.

In order to allow the preparation to come to equilibrium, determinations of catalase activity are usually made several hours after trituration of the tissue. Shorter or longer intervals may, of course, be adopted as standard. The sample used in each determination consists of 1 cc of the thoroughly mixed preparation. This is withdrawn quickly from the bottle with a 1-cc pipette before settling of the agitated suspension can take place. The tip of the pipette may be broken off to facilitate transfer to the suspension. Samples are placed in one arm of each of the 12 tubes which are clamped in place on the support and shaker. Into the other arm of each reaction tube 2 cc of neutralized 12-volume dioxogen is introduced. The stoppers provided with a glass outlet are then firmly fixed to the reaction chambers. Next, the rubber tubing, which remains fastened to the gas bulb, is connected to the stopper and is made to overlap from 3 to 5 cm on the glass outlet. This variation in the length of the connecting tube makes it possible to apply the exact pressure required to bring the arm of the indicator float on the level of the lowest line on the smoked paper. The level of the horizontal arm may itself be adjusted if need be. During these operations the sample and the dioxogen reach the temperature of the bath which is usually held at 20° C. The volume of air in the rubber tubing and above the liquids in the reaction chamber and gas collecting bulb is approximately 75 cc.

When all tubes are connected, the drum is set in motion and the shaker is started as soon as the smoked paper has reached a definite point at which the record of the determination is to begin. A stop watch is used to time the determination from the moment the mixing of dioxogen and the preparation first takes place.

The reaction may be allowed to continue for any desired length of time, usually between 100 and 600 seconds. At the end of a fixed period the horizontal arms of the pen may either be withdrawn from the paper by means of the lever operating the movable support with guide wires, or the drum may be stopped at that time, allowing the further development of gas to be recorded by the height of the vertical line.

After the determination is finished, the reaction tubes are disconnected, washed immediately, and drained thoroughly before they are again placed on the support for the next run. Duplicate or triplicate determinations of each sample can easily be made since only a small quantity of the preparation is required at one time.

Several typical graphs in which variations in catalase activity of apple-tree tissues are clearly shown are reproduced in Figure 3. It should be noted that one set of determinations shown on this figure

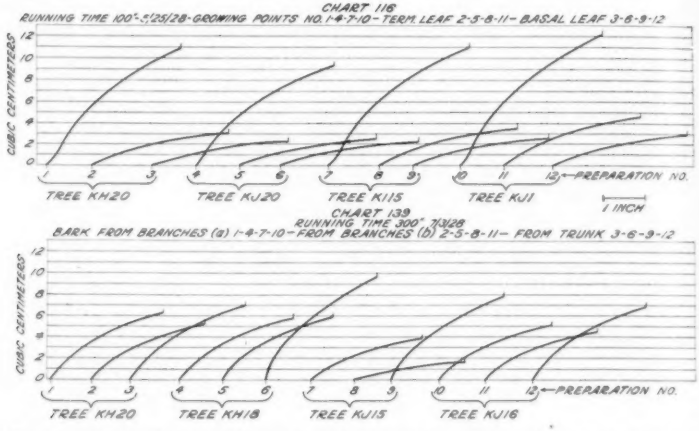

FIGURE 3.—Copies of autographic records of catalase activity. The records on chart 116 are for a run of 100 seconds, and on chart 139, for 300 seconds. Note variation in activity of different tissues and of the same tissue from different trees

lasted for only 100 seconds, the other for 300 seconds. It is obvious that the growing points are far more active than leaf tissue and that bark is less active than either. That the activity for a given time varies with the tree is also clearly shown. By measuring the distance the paper has traveled during a given time, it is easy to translate the results in terms of seconds required to develop a given amount of oxygen. The total amount of gas developed during the entire period is represented by the vertical distance from the base line and may be approximated by directly reading the chart.

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